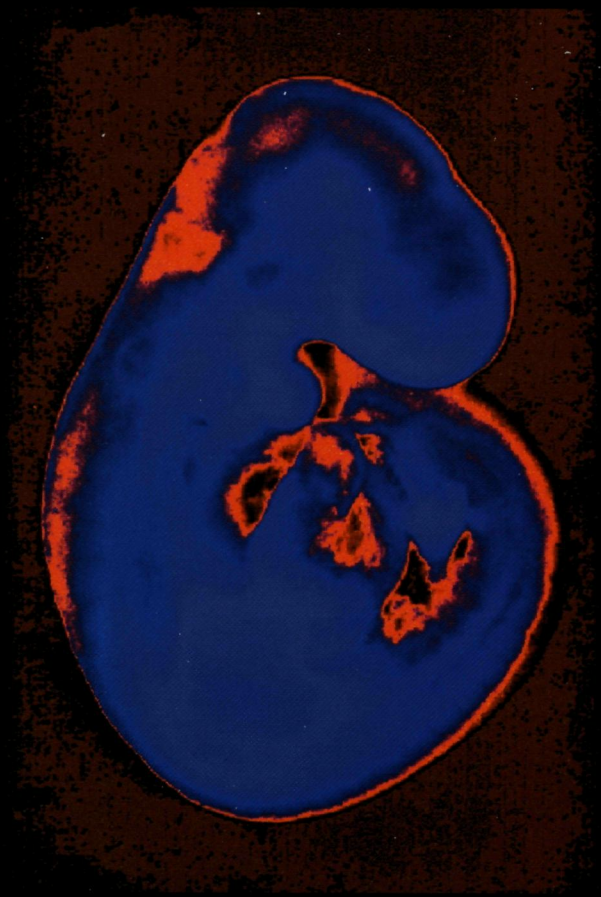
EMBRYOTOXICITY STUDIES ON CYCLOPHOSPHAMIDE AND HOMOCYSTEINE



L.A.G.J.M. van Aerts

EMBRYOTOXICITY STUDIES ON

CYCLOPHOSPHAMIDE AND HOMOCYSTEINE

een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

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Contents

Abbreviations

ALDH	aldehyde dehydrogenase
AUC	area under curve
BSA	bovine serum albumine
CRL	crown-rump length
CV	coefficient of variation
CYP	a specific cytochrome P450 isozyme or its mRNA (terminology according to Nebert *et al.* (1991)
DTT	dithiothreitol
EHNA	erythro-9-[2-hydroxy-3-nonyl]adenosine
5-formylTHF	N^5-formyltetrahydrofolate (folinic acid)
10-formylTHF	N^{10}-formyltetrahydrofolate
GNMT	*N*-glycine methyltransferase
GSH	glutathione
GST	glutathione-*S*-transferase
HL	head length
HPLC	high performance liquid chromatography
HTF	human tubal fluid like
MA	hepatocytes from Aroclor 1254 pretreated male rats
MC	hepatocytes from untreated male rats
MAT	methionine adenosyl transferase
5-methylTHF	N^5-methyltetrahydrofolate
MTHFR	methylenetetrahydrofolate reductase
NTD	neural tube defect
PA	hepatocytes from Aroclor 1254 pretreated pregnant rats
PC	hepatocytes from untreated pregnant rats
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SEM	standard error of the mean
THF	tetrahydrofolate
TMS	total morphological score
WEC	whole embryo culture
YSD	yolksac diameter

1 Objectives And Outline Of Thesis

Objectives

Since the thalidomide disaster the teratogenic risk of chemicals has been recognized as a serious threat to human health. Regulations concerning the introduction of new chemicals have therefore been extended in order to warrant a thorough evaluation of the reproductive toxic risk they might impose to humans. These regulations refer to the use of *in vivo* studies with laboratory animals, usually rats, rabbits and mice. However the use of these animals is costly, time-consuming and has been challenged due to the public debate on the use of animals for experimental goals. For these reasons the search for *in vitro* alternatives is intensified. One of the *in vitro* approaches used in teratologic research is the postimplantation rat embryo culture, or rat whole embryo culture. Since its introduction in teratologic research this model has been applied in several ways. It can be used as a tool to test the embryotoxic potential of chemicals. It is also very useful to investigate mechanisms involved in teratogenesis. To a small extent it has been used to test for pathogenic factors present in sera of women with an abnormal pregnancy outcome.

When chemicals are tested in this culture one has to bear in mind that some chemicals are not embryotoxic in their native form, but only exert an embryotoxic action after they have been bioactivated. The organ that biotransforms the bulk of xenobiotics that enter the body is the liver. This organ is not present in the embryos used in the rat whole embryo culture and the enzymes responsible for the bioactivation of the chemicals are not or only to a very low extent expressed in embryonic tissues. In order to be able to test the embryotoxic potential of such chemicals the rat whole embryo culture has to be combined with an

external biotransforming system capable of bioactivating these chemicals. The development of several approaches of combining adult hepatocytes with rat whole embryo culture and the comparison of them, using cyclophosphamide as a model substance are the objectives of part I of this thesis.

Besides the use of rat whole embryo culture as a way to investigate the embryotoxic potential of chemicals, rat whole embryo culture has been used as a tool in fundamental embryologic and teratologic research as well. In this way this *in vitro* model has been applied in part II of this thesis. The objectives of this part of the thesis were to investigate the embryotoxic potential of homocysteine and elucidate the mechanism of its embryotoxicity. The incentive leading to these studies was the observation that a mild hyperhomocysteinemia frequently occurred in mothers that gave birth to children with neural tube defects (NTDs). This observation indicated that the metabolism of homocysteine in these mothers and possibly their children was deranged. The gestational development of homocysteine metabolism in the rat was therefore investigated in this thesis as well.

Outline of thesis

Almost all of the studies described in this thesis are performed with the rat whole embryo culture model. Part I of this thesis starts with an introductory chapter, shortly reviewing the history of this model and describing the methodology. In *chapter 2* the background of our studies on the use of adult hepatocytes as an external bioactivating system in rat whole embryo culture is clarified. Special attention is given to the metabolism and teratogenicity of cyclophosphamide, that was used as a model substance. *Chapter 3* compares two different approaches: the method to culture adult hepatocytes suspended in the medium in which the embryos are cultured as well - *i.e.* the co-culture model - and the sequential culture model in which embryos are cultured in media in which hepatocytes had been incubated in the presence of a prodysmorphogen. Furthermore the co-culture model was applied to the drug sodium valproate, an anticonvulsant known to increase the risk for spina bifida. It was noted that the sensitivity of the sequential culture model as observed by us differed from that described by others. Therefore in *Chapter 4* we evaluated the effects of gender and Aroclor 1254 pretreatment of the rats providing the hepatocytes on the embryotoxicity and mutagenicity of cyclophosphamide. *Chapter 5* is an extension of the study described in the previous chapter and describes the levels of three main metabolites of cyclophosphamide found in the hepatocytes media. In *Chapter 6* the results and conclusions of the studies of part I are summarized.

In Part II several studies are presented concerning a derangement of homocysteine metabolism and its possible relation to the aetiology of NTDs. In *Chapter 7* the morphogenesis of the neural tube is described. It is indicated multiple pro-

cesses are involved in neurulation, which shows multiple targets exists for teratogens. Special emphasis is given to folate metabolism and its relation to NTDs. Finally in this chapter the incentive that prompted us to study the embryotoxicity of homocysteine is clarified. In *Chapter 8* the embryotoxicity of L-homocysteine in gestational day 10 rat embryos is described. The specificity of the embryotoxic effects of L-homocysteine is demonstrated by comparison with the toxicity of the enantiomere and derivatives of L-homocysteine. Additionally the toxic effect of L-homocysteine to pre-implantation mouse embryos is demonstrated. In *Chapter 9* further details of the embryotoxicity of L-homocysteine to gestational day 10 rat embryos are given and the attenuating effects of vitamin B12, Cu(II)SO4, L-serine, 5-methyltetrahydrofolate and L-methionine are described and discussed. Furthermore this chapter is concerned with the mechanism of L-homocysteine embryotoxicity, giving tentative evidence that inhibition of transmethylation reactions by reduction of the ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH), due to elevated L-homocysteine concentrations, is the cause for L-homocysteine embryotoxicity *in vitro*. Finally, in this chapter the results of experiments with gestational day 9.5 rat embryos are given. These experiments showed that L-homocysteine and L-methionine, but not folinic acid could prevent neural tube defects in these rat embryos, when they were cultured in human serum. In *Chapter 10* the gestational development of homocysteine metabolism in the rat is investigated. In *Chapter 11* the results and conclusions of Part II are summarized. Additionally in this chapter our results and work from other investigators on the mechanisms of folic acid-preventable NTDs are put in perspective and an attempt is made to place present knowledge in an unifying theory. Finally the implications of this theory for the primary prevention of NTDs are discussed.

PART I

THE USE OF ADULT HEPATOCYTES AS AN EXTERNAL BIOACTIVATING SYSTEM IN WHOLE EMBRYO CULTURE

2 Introduction

The culture of postimplantation mammalian embryos

During the postimplantation phase of mammalian embryonic development the main cell lineages diversify and the organ systems are formed. The inaccessibility of the embryos during this period of development in the mother's uterus makes it very difficult to study the processes of development *in utero*. The development of methods to culture these embryos *in vitro* has brought about great advancements in both fundamental embryology and in teratology. The method of culturing postimplantation mammalian embryos is often referred to as *mammalian whole embryo culture* or, depending on the species used, *rat* or *mouse whole embryo culture*.

Methodology

The first attempts to culture mammalian embryos during the postimplantation period were made by Nicholas and Rudnick (1934) in the USA and Jolly and Lieure (1938) in France. Although their results were poor by present standards, they provided a valuable starting point for the development of improved techniques, which was accomplished mostly by the work of New and co-workers (New, 1966; 1978). By the methods they developed it was possible to culture rat embryos from egg cylinder stage (gestational day 8) till the 63 somites stage (gestational day 14.5). However, the embryos explanted at the earliest stages do not develop up to the 63 somites stage *in vitro*. Embryos to be cultured at late stages have also to be explanted at a later stage (Fig. 2.1). The period during which embryos are commonly cultured in teratologic research is from gestational

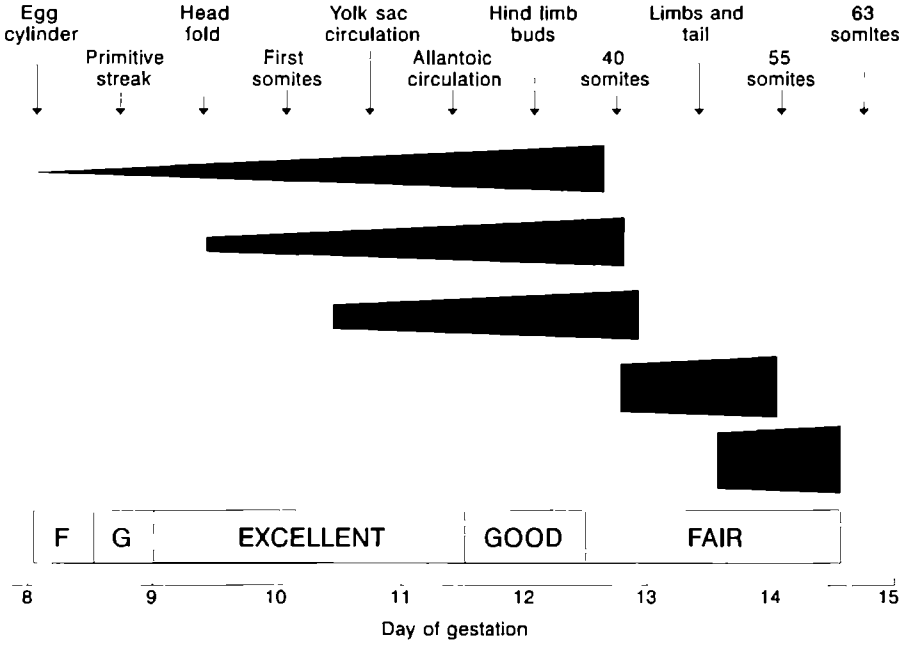

Figure 2.1 Development in culture obtainable from ratembryos explanted at different stages of organogenesis The length of each black area indicates the extent of differentiation in culture. The increasing hight of the black areas from left to right symbolizes the growth in size of the embryos (not drawn to scale). Reprinted from D.A.T New in: Copp and Cockroft (1990), by permission of Oxford University Press. © 1990 IRL Press at Oxford University Press.

day 9.5 (head fold stage) till gestational day 11.5 (appearance of the hind limb buds). During this period development of the embryos *in vitro* is morphologically not different from embryos developed *in utero* (New, 1978). The species used for whole embryo culture are usually rat and mouse. Experiments with other species (opossum, New *et al.*, 1977; hamster, Givelber and DiPaolo, 1968; guinea-pig, Jolly and Lieure, 1938; rabbit, Daniel, 1971 and Glenister, 1971; pig, Trujana and Wrathall, 1985) gave poor results. However more recently fair to good results have been reported on the culture of hamster (Ebron-McCoy *et al.*, 1988) and rabbit embryos (Ninomiya *et al*, 1993). There are several reasons why rodent embryos are more suitable for whole embryo culture. An important factor contributing to the suitability of rodent embryos for whole embryo culture is the presence of an inverted yolksac. As a result of this embryo lies inside its yolk sac. After removal of the Reichert's membrane and the parietal yolk sac, the same layers as in the human conceptus remain, although the layers are still inverted (Garbis-Berkvens and Peters, 1989; Jollie, 1990). In rodents the yolk sac is well developed and is even *in vivo* up till the

twelfth gestational day the main organ exchanging nutrients and gasses between the embryo and the maternal compartment.

The basic features of the methods developed by New *et al.* (1978) are removal of the Reichert's membrane after explantation, but leaving the ectoplacental cone intact, placing the embryos in rotating bottles, using homologous serum as culture medium, and gassing (either continuously or intermittently) with a gas mixture containing varying oxygen concentrations depending on the stage of development (increasing from 5 to 95%). Numerous variations on all aspects of this basic methodology have been made by many authors, which makes comparison of results sometimes difficult. The details of the methods used in this thesis are given in the various chapters of this thesis under the heading *materials and methods*. A comprehensive description of all facets of whole embryo culture can be found in Copp and Cockroft (1990). Although homologous serum is the most optimal medium for development of the embryos *in vitro*, the use of human serum may be advantageous when embryotoxic factors in human blood are investigated. Rat embryos explanted at gestational day 10 (4-8 somites) develop well in human serum, however development of rat embryos in human serum at earlier stages appears to be variable (New, 1990; Priscott 1983; Chatot *et al.*, 1980; Steele, 1985; chapter 9 of this thesis). Addition of a few percent rat serum appears to improve development of these embryos (Anwar and Beck, 1988). Also the addition of 3 mg/ml glucose to the serum appears to be a necessity for the use of human serum as a culture medium.

Embryonic development and its assessment

It has been proposed to name those substances that elicit any toxic effect on the conceptus, including structural or functional abnormalities, *embryotoxic*. *Teratogenicity* on the other hand should be regarded as a special case of embryotoxicity. Substances that cause specific structural or functional abnormalities, usually apparent at birth, within a dose range where there is no generalized toxicity are called *teratogenic* (Sullivan *et al.*, 1993). This terminology has been adopted in this thesis as well[1]. During the optimal period of rat whole embryo culture, *i.e.* from gestational day 9.5 till gestational day 11.5, many organ systems develop rapidly (Brown and Fabro, 1981; Langenfeld *et al.*, 1988). This makes it feasible to the investigator not only to observe embryotoxicity *in vitro*, but to discriminate between general embryotoxicity and teratogenicity in the culture system. Although structural abnormalities are never apparent at birth in this model (the conceptuses will never be born), specific changes in the development of certain parts of the conceptus as a result of a teratogenic insult,

[1]Where necessary previously published papers that have been included in this thesis have been modified according to this terminology

	Score	0	1	2	3	4	5
A	Yolksac circulation	No visible blood islands	Blood islands present	Vitelline vessels and few yolksac vessels present Circulation starting	Full yolksac plexus of vessels Circulation vigorous	Yolk stalk obliterated Vitelline artery and vein well separated	
B	Allantois	Free in exocoelom	Fused with chorion	Umbilical vessels present No circulation	Circulation trough umbilical vessels		
C	Flexion	Ventrally convex	Turning 25% complete	Turning 50% complete	Turning 75% complete	C shaped, dorsally convex	Spiral shaped
D	Heart	Endocardial rudiment straight No heart beat	Endocardial rudiment S shaped Heart beat started	Convoluted cardiac tube	Bulbus cordis, atrium commune and ventriculis communis present	Dividing atrium commune	
E	Caudal neural tube	Neural plate flat or neural folds only starting to elevate	Almost closed at level of somite 2	Fused at level of somite 4/5	Caudal neuropore still open	Caudal neuropore closed	
F	Rhombencephalon	Flat neural plate	Neural folds elevating	Neuropore formed, but still open	Neuropore closed	Pronounced pontine flexure	
G	Mesencephalon	Flat neural plate	Neural folds elevating	Neural folds approaching, but not fused	Neural folds fused	Division between mesencephalon and diencephalon visible	
H	Prosencephalon	Flat neural plate or neural folds just starting to elevate	Neural folds approaching but not fused	Completely fused prosencephalon	Telencephalic evaginations visible	Well elevated telencephalic hemispheres	
J	Otic system	No sign of otic development	Flattened or indented otic primordium	Otic pit	Otic cyst almost closed	Otic cyst completely closed	Dorsal recess visible but smaller than cyst
K	Optic system	Not visible	Very thin line	Sulcus opticus clearly visible	Sulcus opticus elongated	Open eye stalk clearly visible	
L	Olfactory system	Not visible	Olfactory plate	Olfactory plate with rim			
M	Branchial bars	None visible	I visible	I and II visible	I, II and III visible	II overgrowing and obscuring III	
N	Maxillary process	Not visible	Concave cleft	Flat or convex cleft			
P	Mandibular process	Brachial bars I well separated	Bars joined, but not fused	Branchial bars I fused			
Q	Fore limb buds	Not present	Height 25% of width 'torso'	Height 50% of width 'torso'	Height same as width torso		
R	Hind limb buds	Not present	Height 50% of width 'torso'	Height bigger than width 'torso			
S	Somites	0 6	7 13	14 20	21 27	28 34	35 41

Table 2.1 Morphologic scoring system Adapted from Brown and Fabro (1981)

A1	Yolksac circulation	defective
A2		small[1] yolksac vessels
A3		small[1] or avascular
B1	Allantois	not fused with chorion
B2		large[1]
B3		small[1] or avascular allantois
B4		Fused abnormally
C1	Flexion	incomplete
C2		absent
C3		inverted tail (positioned at wrong side of head)
D1	Heart	delayed cardiac tube formation
D2		wide[1] pericardial sac, filled with fluid
D3		other abnormalities (specify)
E1	Caudal neural tube	Open posterior neuropore (at a stage where it should be closed)
E2		irregular dorsal midline
E3		aplasia
E4		kinky
E5		locally wider
E6		anterior-posterior fusion
E7		curving more extreme
F1	Rhombencephalon	open rhombencephalon
F2		large[1] or transparent rhombencephalon
G1	Mesencephalon	open mesencephalon
H1	Prosencephalon	open prosencephalon
H2		small[1] prosencephalon
He1	Other head region	irregular suture line
He2		abnormal craniofacial appearance
He3		haemorrhages
He4		edema
J1	Otic primordium	deformed
K1	Optic primordium	deformed
M1	Branchial bars	one pair or more missing
N1	Maxillary processes	large[1]
N2		small[1]
P1	Mandibular processes	unapproached[1]
Fa1	Other facial region	(specify)
Q1	Forelimb buds	small[1]
Q2		missing
S1	Somites	small[1]
S2		irregular
S3		fused
S4		amorphogenesis
Oth1	Others	edema
Oth2		haemorrhages
Oth3		general growth retardation
Oth4		embryonic death
Oth5		cell death[2]
Oth6		other abnormalities (specify)

Table 2.2 Checklist of dysmorphogenic features. [1]As compared to the normal situation. [2]Visible as opaque regions or spots at places where these are normally not present.

without an effect on other parts of the conceptus, may be regarded as an expression of the teratogenic potential of a substance (Schmid and Cicurel, 1986).

To assess embryonic development during culture and to quantify the extent to which it is impaired, several scoring systems have been developed, of which the one developed by Brown and Fabro (1981) for the evaluation of rat embryo culture is the most elaborate. In an adapted form this scoring system is used in this thesis as well (Table 2.1). Adding up all scores in this system for each embryo results in the *Total Morphological Score* (TMS), which is an accurate and sensitive measure for the embryonic development that occurred during culture. However teratogenic effects affecting only one or two of the separate scores may be masked in this overall measure. One might be tempted to statistically test for differences between control and experimental embryos for each separate morphological parameter. However the very large number of comparisons made in such a way makes this precarious.

Besides the use of the morphological scoring system all abnormal features of the embryos were recorded as well. For this purpose a check list was used (Table 2.2). Often such abnormal features are called *malformations*. However it has been argued that abnormalities visible at gestational day 11 or 11.5 do not necessarily have to result in malformations apparent at birth. Therefore these abnormalities should rather be called *dysmorphogenic features* instead of malformations (Brown *et al.*, 1990). This terminology has been adopted in this thesis.

Finally three growth-related parameters were measured as well, namely head length, crown-rump length and yolksac diameter.

Prodysmorphogens in whole embryo culture

The principal usage of whole embryo culture is to test the teratogenic or embryotoxic potential of compounds. A major difference with *in vivo* testing is the exclusion of the influence of maternal factors. This gives the investigator the ability to examine the direct effects of the compound tested. An important maternal factor having the potential of affecting the embryotoxic effect of a compound is the maternal metabolism of the xenobiotic, usually called biotransformation. When this biotransformation leads to the formation of metabolites that do elicit toxic effects, this process is often named bioactivation. Many substances do not cause any embryotoxic or teratogenic effects in their parent form, but first have to be bioactivated (Juchau, 1989). Similarly compounds do often not show mutagenic or carcinogenic properties unless they have been bioactivated. Such compounds are referred to as promutagens or procarcinogens. It has therefore been proposed to name compounds that have to be bioactivated before they elicit embryotoxic or teratogenic effects proteratogens (Oglesby *et*

al., 1986). However, referring to the terminology adopted above, teratogenicity comprises only a part of the embryotoxic spectrum. Morphologic abnormalities observed after exposure of embryos to embryotoxic substances are called dysmorphogenic features. In conjunction with this terminology, substances that elicit these abnormalities should therefore be named *dysmorphogens*. From this it follows that substances that need to be bioactivated before they elicit dysmorphogenic features should be called *prodysmorphogens*.

If whole embryo culture is used without taking into account the possible effects of biotransformation for (pre-)screening of compounds, the exclusion of maternal biotransformation might be judged as a disadvantage. However biotransformation of xenobiotics is quite often different in laboratory animals from biotransformation in humans. Therefore, bearing in mind that compounds are usually tested to investigate their potential harmfulness for humans, the results obtained with *in vivo* testing in laboratory animals may rather be misleading instead of predicting when it are the metabolites that cause the harmful effects. Testing the parent compound and species-specific metabolites separately in whole embryo culture may therefore provide a much better insight in the embryotoxic potential of substances.

External bioactivating systems

The metabolites possibly involved in the embryotoxicity of a compound are often unknown or these metabolites are unavailable. It is therefore necessary to develop other approaches to investigate the role of maternal metabolism in the embryotoxicity of compounds. Basically there are two ways to introduce species-specific metabolites in the whole embryo culture system when they are not directly at hand. One is to administer the parent compound to an animal and draw the blood from it in which the metabolites formed by the animal are present. Subsequently the serum may be used to culture the embryos in. This may be especially interesting when not the blood from an animal is used but human blood from for example a patient who has taken a drug. In such a case human metabolites of the drug can be tested in whole embryo culture, as first was demonstrated by Chatot *et al.* (1980). A drawback of this approach is the loss of instable metabolites which may occur as a result of the necessity to heat-inactivate the serum and the occurrence of extended time periods between the moment of administration of the drug, the drawing of the blood and the moment of testing in the embryo culture.

An alternative way to introduce species-specific metabolites in the whole embryo culture system is by adding an external bioactivating system to the culture. The first to use this approach were Fantel *et al.* (1979) and Sanyal *et al.* (1979), who showed that the *in vitro* embryotoxicity of cyclophosphamide could be greatly increased by adding a rat hepatic microsomal fraction to the embryo culture.

The presence of reconstituted microsomes and the necessary cofactors is tolerated by the embryos in culture (Kitchin *et al* , 1981a,b) However there is concern about artifacts such as prolonged exposure of differentiating tissues to stable derivatives that are not detoxified in reconstituted systems and the persistence of entirely unphysiological reaction products (Welsch, 1992) Also the spectrum of reactions catalyzed by microsomes *in vitro* is limited Inactivation of toxic reaction products may occur in living animals or intact liver cells but be absent in reconstituted microsomal fractions Furthermore toxic products may be retained in the hepatocytes and never reach the embryo *in vivo* The shortcomings of the use of microsomes have prompted Oglesby *et al* (1986) to develop a co-culture system of intact hepatocytes and rat embryos In the model they developed, hepatocytes of phenobarbital pre-treated and untreated rats, rabbits and hamsters were used They showed that the embryotoxicity of cyclophosphamide could be modified to different degrees depending on the pre-treatment and the species from which the hepatocytes originated Instead of co-culturing hepatocytes and rat embryos by cultivating the embryos on top of a monolayer of hepatocytes, co-cultivation is also possible by suspending the hepatocytes in the serum in which the embryos are floating, as was shown by Piersma *et al* (1991) A somewhat different approach of combining intact hepatocytes and rat embryos was undertaken by Bechter *et al* (1989) In the model they developed cyclophosphamide was added to a monolayer culture of rat hepatocytes Medium samples from this culture were taken at various intervals and used for the culture of postimplantation rat embryos to assess the embryotoxicity of the metabolites formed Since the hepatocytes and the embryos are not cultured together, but sequentially, this model is referred to as the *sequential culture model* An advantage of the sequential culture model over the co-culture models is that some aspects of the pharmacokinetics can be taken into account as well, since the appearance and disappearance of embryotoxic metabolites can be monitored in this model

Further evaluation of the sequential culture model originally described by Bechter *et al* (1989) and the co-culture model described by Piersma *et al* (1991) is the main objective of this part of this thesis The results of these investigations will be presented in the next three chapters

Cyclophosphamide

Cyclophosphamide (Fig 2 2) has now been used for over three decades as an anti-neoplastic and immunosuppressive drug A well-known characteristic of this drug is that it first must be bioactivated before it is therapeutically effective The necessity for bioactivation is not only true for its therapeutic action but also the toxic properties of cyclophosphamide only become apparent after bioactivation For this reason cyclophosphamide has been used extensively in

toxicologic studies as a model substance to demonstrate the presence of biotransforming capacity of tissues, cells or cell-free extracts used in *in vitro* models. In this way cyclophosphamide has also been applied in our studies. Since model-dependent differences in metabolism of cyclophosphamide were part of the subject of our studies and the goal was the application of bioactivating systems in whole embryo culture, both the metabolism and the teratogenicity of cyclophosphamide will be shortly described below.

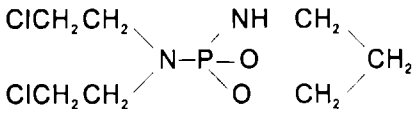

Fig. 2.2 Structural formula of cyclophosphamide

Metabolism

The metabolism of cyclophosphamide as described here is schematically represented in Fig. 5.2. The first step of its bioactivation is a hydroxylation at the ring C-4 position, which yields 4-hydroxycyclophosphamide. This reaction is performed by NADPH-dependent mono-oxygenases (Sladek, 1988). There is evidence this reaction may also be catalyzed by peroxidases, however the contribution of these enzymes to the formation of 4-hydroxycyclophosphamide in hepatic tissues is neglectable due to the abundance of mono-oxygenases in these tissues (Kanekal and Kehrer, 1993). The mono-oxygenases catalyzing the oxidation of cyclophosphamide to 4-hydroxycyclophosphamide are enzymes belonging to the cytochrome P450 family. In untreated rats the principal forms of cytochrome P450 responsible for this activation are CYP2C6 and the male-specific CYP2C11. However when rats are phenobarbital pretreated CYP2B1 is the main catalyst (Clarke and Waxman, 1989).

Once cyclophosphamide is bioactivated to the 4-hydroxy form it can tautomerize to an open ring structure, that is aldophosphamide. Aldophosphamide is unstable in an aqueous solution and will spontaneously degrade to phosphoramide mustard by ß-elimination of acrolein. These two reaction products are believed to be responsible for most toxic effects caused by cyclophosphamide. Subsequently phosphoramide mustard will degrade spontaneously to another mustard compound, namely nornitrogen mustard (Jardine *et al.*, 1978).

Above the metabolic steps necessary for cyclophosphamide activation are summarized. However besides activation other metabolic steps are possible as well, notably those that lead to detoxification. Detoxification of cyclophosphamide may occur in two different ways. One is by oxidative reactions that render less or non-toxic metabolites, and the other is conjugation with glutathione (GSH). Both the main forms of 'activated' cyclophosphamide - 4-hydroxycyclophosphamide and aldophosphamide - are prone to oxidative detoxifications.

The first can be oxidized to 4-ketocyclophosphamide, probably by an aldehyde oxidase (Hohorst *et al.*, 1971), and the second to carboxyphosphamide, a reaction that is catalyzed aspecifically by numerous aldehyde dehydrogenases (Domeyer and Sladek, 1980; Lindahl, 1992). Conjugation with GSH may occur spontaneously or mediated by specific enzymes, named glutathione-*S*-transferases (GSTs). GSH conjugation of the native cyclophosphamide molecule occurs only enzymatically at a neutral pH by displacement of the chloride atoms, whereas phosphoramide mustard may react both spontaneously and by mediation of GSTs through an aziridinium intermediate (Colvin *et al.*, 1993). Acrolein may react also with GSH which results in the formation of glutathionylpropionaldehyde. The latter reaction is reversible and acrolein may also spontaneously react with other thiol compounds (Ohno and Ormstadt, 1985).

Above the role of GSH and GSTs in the detoxification of cyclophosphamide and its metabolites is explained. However, the role of GSH and GSTs in the biotransformation of cyclophosphamide has a more ambivalent nature. Besides in detoxification they also have a function in the stabilisation of 'activated' cyclophosphamide. As explained above 'activated' cyclophosphamide, once it is formed, spontaneously degrades to phosphoramide and acrolein. However, as already pointed out by Draeger *et al.* (1976) and Peter *et al.* (1976), substitution of the 4-hydroxy group with a thiol compound will result in a thiol conjugate of medium stability in an aqueous solution. The most abundant thiol present in hepatocytes is GSH. GSH reacts either with the dehydrated form of 4-hydroxycyclophosphamide - 4-iminocyclophosphamide - or 4-glutathionylcyclophosphamide is formed via a hemithioacetal intermediate (Pallante *et al.*, 1986; Lee *et al.*, 1991). Since this conjugate is of medium stability in an aqueous solution it will spontaneously deconjugate after a while. In this way 'activated' cyclophosphamide is formed once again. This process has therefore also been referred to as 'delayed toxication' (Hohorst *et al.*, 1976). The hydrophilic properties and relative stability of this conjugate make it likely that it has an important role in the redistribution of 'activated' cyclophosphamide (Fenselau *et al.*, 1982; Dirven, 1994).

Teratogenicity

The teratogenicity of cyclophosphamide is well investigated in laboratory animals, mostly rats, mice and rabbits. The most predominant congenital malformations induced are malformations of the central nervous system and skeletal deformities. In monkeys (*Macaca mullata)* similar results were obtained (Mirkes, 1985). Experience on the teratogenicity of cyclophosphamide in man is obtained with cancer patients on therapy. Multiple congenital malformations have been described frequently. However in most cases chemotherapy was combined with irradiation therapy and in one case a multiagent chemotherapy was used. It

is therefore difficult to draw conclusions from these observations (Mirkes, 1985; Ostensen, 1992). A case of transplacental secondary cancer in a twin exposed *in utero* has been reported as well (Zemlickis *et al.*, 1993).

The mechanism of teratogenicity of cyclophosphamide has been studied intensively. In the beginning it was thought that - contrary to the mutagenic and antineoplastic action of cyclophosphamide - the teratogenicity of cyclophosphamide was caused rather by the parent compound than by metabolites thereof (Gibson and Becker, 1968). However as Fantel *et al.* (1979) and Sanyal *et al.* (1979) demonstrated, the *in vitro* embryotoxicity of cyclophosphamide could be greatly increased by adding a reconstituted microsomal enzyme system to the culture. Subsequent studies in which metabolites were added to the embryo culture or where conceptuses were injected intra-amniotically *in vivo*, showed that phosphoramide mustard and acrolein were the proximate teratogens (Mirkes *et al.*, 1985; Juchau, 1989; Welsh, 1992).

Although the congenital malformations induced by acrolein and phosphoramide mustard are very much alike, the mechanisms seem to differ. Whereas the proteins of the yolk sac seem to be the prime target of acrolein, phosphoramide mustard seems to react primarily with the embryonic DNA (Welsh, 1992). The interaction of phosphoramide mustard with embryonic DNA has been given the most attention and a number of facts have been established on this matter. Adducts of guanine and breakdown products of phosphoramide mustard have been identified in organogenesis-stage rat embryos after exposure *in vitro* (Mirkes *et al.*, 1992). Exposure of rat embryos to 'activated' cyclophosphamide induces both single strand DNA breaks and DNA cross-linking (Little and Mirkes, 1987). Phosphoramide mustard induces dose-dependently a perturbation of the cell cycle, with a decrease in the percentage of cells in G_1/G_0, a delay in S-phase, and an increase in the percentage of cells in G_2/M (Little and Mirkes, 1992). Recently Chen *et al.* (1994) demonstrated that a teratogenic dose of phosphoramide mustard caused DNA fragmentation and increased expression of sulphated glycoprotein-2, suggesting apoptosis is involved in mediating the teratogenicity of phosphoramide mustard.

Co-Culture And Sequential Culture Of Maternal Hepatocytes And Post-Implantation Rat Embryos[1]

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3

Introduction

The whole embryo culture (WEC) of rodent embryo's as developed by New (1978) has been used now for over ten years to study the embryotoxicity of substances either as a safety evaluation test (Schmid and Cicurel, 1986) or to study mechanisms of teratogenesis (Freeman and Steele, 1986). Since several substances require bioactivation in order to produce embryotoxic effects, it is a major drawback of the WEC that the biotransforming capacity of the embryos is very limited (Wilson, 1978). Therefore several approaches have been used to introduce an active adult hepatic biotransforming system to the WEC. Microsomal fractions (Kitchin *et al.* 1981a; 1981b; Fantel *et al.*, 1979) or liver homogenates contain only part of the metabolic activity, which results in different metabolic patterns when compared with intact hepatocytes (Oglesby *et al.*, 1986). Several methods have been developed to combine the WEC with a culture of intact hepatocytes (Brown and Kram, 1982; Oglesby *et al.*, 1986; Bechter *et al.*, 1989; Piersma *et al.*, 1991). In these reports cyclophosphamide has proven to be a useful prodysmorphogen in order to show the efficacy of the model under investigation. In our study we therefore used cyclophosphamide to compare co-culture and sequential culture of maternal hepatocytes and post-implantation rat embryos in human serum.

Valproic acid, an anti-epileptic drug, is associated with an increased risk of

[1] See note on p. 9

developing spina bifida (as high as 1-2%) (Lammer *et al*, 1987, Anonymous, 1988) The parent substance is rapidly metabolized (Nau, 1986, Binkerd *et al*, 1988, Rettenmeier *et al*, 1986) and metabolites differ widely in teratogenic potence (Nau and Loscher, 1986) We therefore used the co-culture system to investigate wether there was a potentiating effect of the metabolic system when sodium valproate was added

Materials and methods

Serum preparation

Blood was taken from adult volunteers (male and female) between 20 and 50 years of age The blood was defibrinized during donation by shaking with glass pearls in a glass bottle The defibrinized blood was centrifuged immediately and the serum was heat-inactivated The glucose concentration was raised by 4 mM After testing each individual serum for compatibility with WEC, the serum of two to four individuals was pooled and stored at -20 °C

Embryo culture

Random-bred Wistar rats were housed in pairs or with three together in cages and were allowed to eat (standard food, Hope farms) and drink (tap water) *ad libitum* Lights were on from 12 00 a m till 12 00 p m On day 0 females (11 26 weeks old, 200 300 g), willing to mate, were brought together with males (1 1) from 9 00 a m till 12 00 a m Embryos were explanted between 1 30 p m and 3 30 p m on day 10 Only embryos between four and eight somites and without any abnormalities were used for WEC The embryos were cultured for 24 hours in a 25 ml glass bottle containing 2 ml medium at 37 5 °C and rotated at 30 rpm The culture bottles were previously gassed with a gas mixture of O2, N2 and CO2 (5 90 5) After 2 and 18 hours of culture the bottles were gassed with O2, N2 and CO2 (20 75 5)

Hepatocyte isolation and co-culture of hepatocytes and embryos

Hepatocytes were isolated from 10 days' pregnant rats by the perfusion method This method and the co-culture system have been described previously (Piersma *et al*, 1991), except for the perfusion buffers being Hank's balanced salt solution instead of Ham's F10 and the co-culture medium being human serum instead of rat serum Sodium valproate or cyclophosphamide was added to the medium in 0 1 ml Hank's balanced salt solution

Sequential culture of hepatocytes and embryos

Hepatocytes, isolated as indicated above, were suspended in supplemented Williams' medium E (Bechter *et al*, 1989) with 10% foetal calf serum (FCS) at a concentration of 8×10^5/ml Cells were seeded on rat-tail collagen coated petri dishes (10 cm ø, Nunc), 10 ml/plate and incubated at 37 °C with 5% CO2 in air and with 100% humidity After 4 hours the medium was replaced by supplemented Williams' medium E without FCS, with or without 1 mM cyclophosphamide After 0, 1, 2, 4, 8 and 16 hours plates were taken, the medium underwent sterile filtration, and aliquots were frozen in fluid N2 and stored at -80°C For embryo culture 0 8 ml of the hepatocyte culture medium was added to 1 2 ml human serum and embryos were cultured as indicated above

Morphological endpoints

During the culture the embryos were monitored inside the incubation chamber for turning, neural tube closure, heart rate and yolksac circulation This was done after 2 and 18 hours of culture and at the end of the culture After culture the embryos were scored as described by Brown and Fabro (1981), resulting in a total morphological score (TMS) Yolksac diameter (YSD), crown-rump length (CRL), head length (HL) and increase in number of somites (Δ somites) were also measured All dysmorphogenic features were recorded

Statistical analysis

The least square means of the morphological parameters of each group were estimated by analysis of covariance, taking the number somites at the start of the culture as covariant Least square means were compared using Student s t-test Differences were regarded as statistically significant at $P<0\ 05$

Results

Co-culture of hepatocytes and embryos cyclophosphamide

In the presence of hepatocytes and 100 μM cyclophosphamide or more Δ somites was significantly smaller compared with controls (0 μM cyclophosphamide) (Fig 3 1e) Also the number of dysmorphogenic features/conceptus was significantly increased under these conditions (Fig 3 1f) TMS and HL were significantly decreased at a concentration of 330 μM cyclophosphamide or more (Fig 3 1a,c) CRL was also significantly decreased at this concentration, but was not at 1mM cyclophosphamide (Fig 3 1b) YSD was not changed at any concentration tested

In the absence of hepatocytes at the highest concentration of cyclophosphamide (3 3 mM), TMS decreased significantly (Fig 3 1a) and the number of dysmorphogenic features/conceptus increased significantly (Fig 3 1f) Other morphological parameters didn't differ significantly at any concentration of cyclophosphamide tested compared with controls (0 μM cyclophosphamide)

Dysmorphogenic features appearing in a dose-dependent way as a result of incubation of the embryos and hepatocytes with cyclophosphamide were abnormal craniofacial appearance, dysmorphogenesis of the caudal part of the neural tube and cell death

Co-culture of hepatocytes and embryos sodium valproate

The presence of hepatocytes did not grossly affect the embryotoxicity of sodium valproate (Fig 3 2) Most morphological parameters were significantly decreased at 3 mM sodium valproate (Fig 3 2a e) The number of dysmorphogenic features/conceptus was significantly increased at 2 mM sodium valproate (Fig 3 2f) Absence of malformations at low concentrations sodium valproate (0 3 and 1 mM) in the presence of hepatocytes is notable, however,

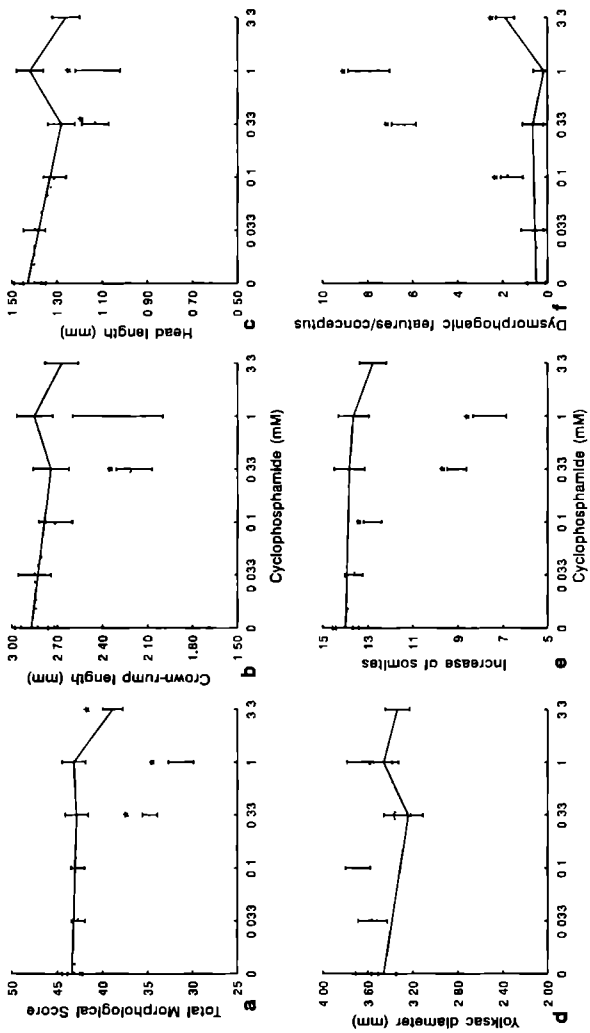

Fig. 3.1. Effects of cyclophosphamide on TMS (a), CRL (b), HL (c), YSD (d), Δ somites (e) and number of dysmorphogenic features/conceptus (f) of rat whole embryos *in vitro* in the presence (—) or absence (—) of maternal hepatocytes Least square means ± SEM n = 6-8 * = $P<0.05$ (Student's t-test)

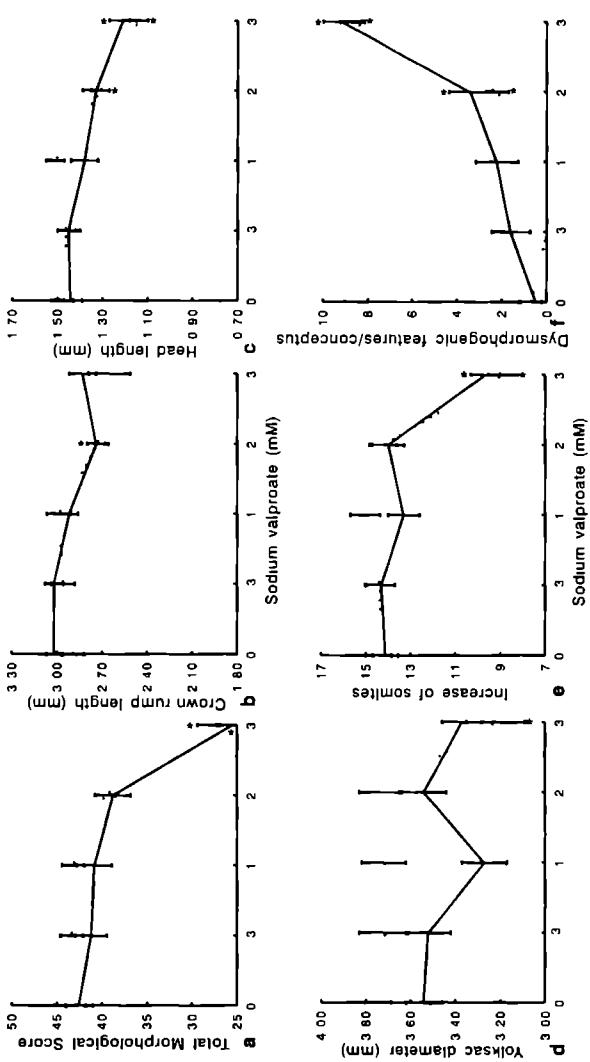

Fig 3 2 Effects of sodium valproate on TMS (a), CRL (b), HL (c), YSD (d), ▵ somites (e) and number of dysmorphogenic features/conceptus (f) of rat whole embryos *in vitro* in the presence (—) or absence (——) of maternal hepatocytes Least square means ± SEM n = 6 8 * = P<0 05 (Student s t test)

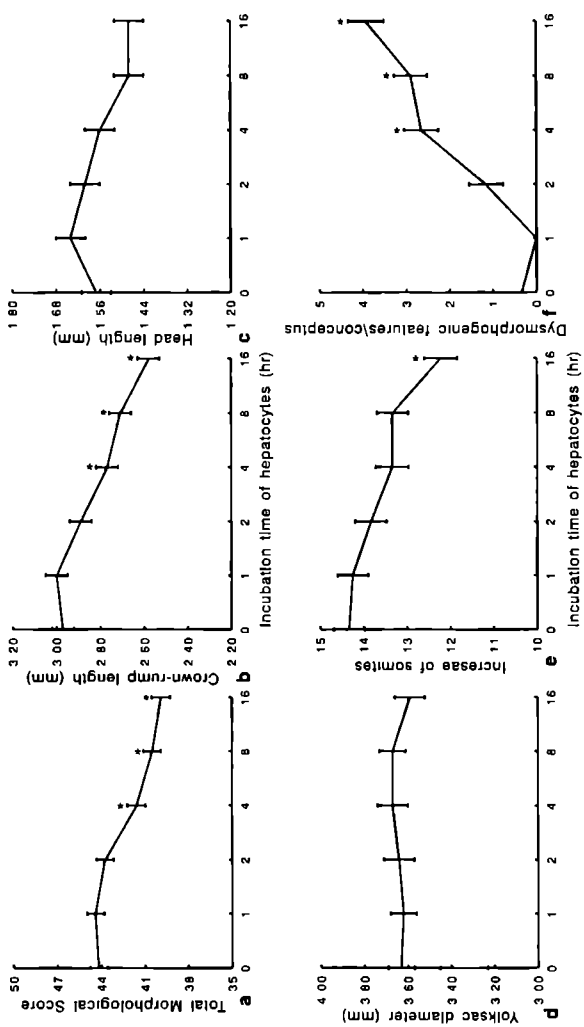

Fig. 3.3. Effects of cyclophosphamide on TMS (a), CRL (b), HL (c), YSD (d), ▵ somites (e) and number of dysmorphogenic features/conceptus (f) of rat whole embryos *in vitro*, using medium in which hepatocytes have been cultured for varying periods Least square means ± SEM n = 6-8 * = P<0 05 (Student's t-test)

dysmorphogenic features did occur at these concentrations when hepatocytes were absent. This difference was only statistically significant at 0.3 mM sodium valproate. However, as mentioned before, a comparison of the embryos cultured without hepatocytes with 0.3 mM sodium valproate and there controls (0 mM sodium valproate) showed no significant increase of dysmorphogenic features/conceptus. At further analysis of the types of dysmorphogenic features, dose-dependent impairment of yolksac circulation, irregular suture line of the neural tube, delay in limb bud formation, irregular somites and cell death were seen. At 3 mM sodium valproate many more dysmorphogenic features could be seen, such as malformed optic and otic vesicles, open cranial neural tubes, incomplete (or absence of) turning and growth retardation.

Sequential culture of hepatocytes and embryos: cyclophosphamide.

Fig. 3.3 shows that after 4 hours of incubation of the hepatocytes in the presence of 1 mM cyclophosphamide, enough embryotoxic metabolites have been formed to impair the normal growth of the embryos. TMS and CRL are significantly decreased from this time onward (Fig. 3.3a,b). The number of dysmorphogenic features/conceptus has also significantly increased at that time (Fig. 3.3f). HL and YSD are not affected to a significant degree (Fig. 3.3c.d). The types of dysmorphogenic features are the same as those seen in the co-culture system with cyclophosphamide.

Discussion

The results obtained with the co-culture system when cyclophosphamide was tested are in accordance with those described earlier (Piersma *et al.*, 1991). Hepatocytes are active, embryos grow well in the presence of hepatocytes, and bioactivation of cyclophosphamide occurs when hepatocytes are co-cultured with the embryos. This demonstrates that the use of human serum is compatible with the co-culture system.

Inconsistency of CRL as a morphological parameter when cyclophosphamide or sodium valproate are tested in the co-culture system must be explained by the low number of surviving embryos at high concentrations of test substance added and the interference of these substances with the turning of the embryos that did survive. YSD seems to be an insensitive parameter. We therefore plead in favour of the use of more elaborate parameters such as the TMS originally described by Brown and Fabro (1981).

The absence of dysmorphogenic features in the presence of hepatocytes and low concentrations of sodium valproate (whereas dysmorphogenic features did occur when hepatocytes were absent) suggests some detoxifying effect on the part of the hepatocytes. The fact that no potentiating effect by the hepatocytes could be observed, means that either potent embryotoxic metabolites are not

formed in quantities large enough to cause any increase in embryotoxicity, or that the formation of embryotoxic metabolites is equalled by the disappearance of sodium valproate, which is embryotoxic itself.

Our results demonstrate that it is possible to bioactivate cyclophosphamide and, thereby, elicit its embryotoxic potencies by culturing hepatocytes and embryos sequentially. In contrast to an earlier report (Bechter *et al.*, 1989), we could not detect a significant embryotoxic effect within 4 hours of incubation of the hepatocytes. This difference may be explained by the fact that we used non-induced maternal hepatocytes, whereas Bechter and coworkers used Aroclor 1254-induced male hepatocytes. This also explains why we needed much higher concentrations of cyclophosphamide and that embryotoxic compounds are still present in the hepatocytes medium after 16 hours; this, too, is in contrast with Bechter's results, who used only 60 μM cyclophosphamide and found a maximum embryotoxicity after 5 hours. It is therefore clear that the sequential culture system we used is less sensitive than the one described by Bechter, but it also demonstrates that under the more physiological circumstances of our system bioactivation of cyclophosphamide and embryotoxicity do still occur.

When we compare the results of the co-culture system and the sequential culture system, it is obvious that the sequential culture system is as sensitive or slightly less sensitive than the co-culture system. (0.33 mM cyclophosphamide in the sequential culture system showed some embryotoxicity after 16 hours of incubation of the hepatocytes; results not shown). It therefore seems that the loss of some of the bioactivating capacity during the attaching period of the hepatocytes and the loss of instable metabolites are not of great importance under these circumstances. Compared with the co-culture system, the sequential culture system has two major advantages: (i) One hepatocyte isolation and incubation provides sufficient medium for all embryo cultures of an experiment, whereas in the co-culture system, hepatocytes must be isolated for each embryo culture. (ii) Taking medium from the hepatocyte culture at different times gives an impression of the time-dependent formation of embryotoxic metabolites and detoxification of embryotoxic compounds.

In conclusion, we believe that both the co-culture system and the sequential culture system can be useful in eliciting the embryotoxicity of metabolites formed from suspected prodysmorphogens. However the sequential culture system seems to be the more practical approach.

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Sex Difference In Aroclor 1254 Induction Of Rat Hepatocytes: Consequences For *In Vitro* Embryotoxicity And Mutagenicity Of Cyclophosphamide[1]

4

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Toxicology in vitro 7:769-775

Abstract The sequential culture of rat hepatocytes and postimplantation rat embryos has been proposed as a model for the *in vitro* testing of prodysmorphogens. Comparing this model with a model in which embryos and hepatocytes are cultured simultaneously a striking difference in sensitivity was noted. To address the question whether this difference could be explained by different sex and/or Aroclor 1254 pretreatment of the rats providing the hepatocytes, an experiment was designed with four groups: male Aroclor 1254 pretreated (MA), male untreated, pregnant female Aroclor 1254 pretreated (PA) and pregnant female untreated rats. Hepatocytes were incubated in the presence of cyclophosphamide and rat embryos were cultured in the media derived from the hepatocyte culture (*i.e.* the sequential culture model) Additionally, the cyclophosphamide concentrations of the media were analysed and subsequently the media were tested in a bacterial mutagenicity test (*Salmonella typhimurium* TA 1535). With a cyclophosphamide concentration of 300 μM, MA produced maximum embryotoxicity and mutagenicity after 4 hours of hepatocytes incubation. All other groups showed no or only a slight increase in embryotoxicity and mutagenicity for all hepatocyte incubations. MA was also quickest to eliminate cyclophosphamide from the medium These results indicate that despite a strong increase in total cytochrome P450 in both sexes as a result of Aroclor 1254 pretreatment, and in the absence of a significant difference in total cytochrome P450 between MA and PA, Aroclor 1254 pretreatment has a much more pronounced effect in male rats than in pregnant female rats with regard to the production of embryotoxic and mutagenic metabolites of cyclophosphamide.

Introduction

Some substances require bioactivation before they can exert their embryotoxic

[1]See note on p. 9

activity It is therefore a major drawback of isolated whole embryos in culture that their biotransforming capacity is limited In order to facilitate biotransformation in whole embryos in culture, several approaches have been used Besides addition of hepatic microsomal fractions (Fantel *et al*, 1979, Kitchin *et al*, 1981a,b) intact hepatocytes have also been used, both in co-culture (Oglesby *et al*, 1986, Piersma *et al*, 1991, VanAerts *et al*, 1992) and in sequential culture (Bechter *et al*, 1989, VanAerts *et al*, 1992) with rodent embryos In the sequential culture model hepatocytes are incubated with the substance under investigation, whereafter the medium containing metabolites is mixed with serum and rodent embryos are cultured in it

When we compared our results, obtained with the sequential culture model, using cyclophosphamide as prodysmorphogen (VanAerts *et al*, 1992), with the results obtained by Bechter *et al* (1989), we noted a striking difference in sensitivity This difference could not simply be explained by the fact that Bechter and coworkers had pretreated their (male) rats with Aroclor 1254, since pretreatment with Aroclor 1254 of our (pregnant female) rats did not show a potentiating effect on the bioactivation of cyclophosphamide (L A G J M VanAerts, unpublished results, 1991) We therefore designed an experiment in which both sex and pretreatment with Aroclor 1254 of the rats providing the hepatocytes were varied, in order to investigate the role of both factors in their capacity to influence the bioactivation of cyclophosphamide

Materials and methods

Hepatocyte culture

Male random bred Cpb WU (Wistar) rats (8-10 wk old, 220-280 g) were housed in groups and were allowed access to food (MRH-TM pellets, Hope farms B V, Woerden, The Netherlands) and water [acidified tap water (0 025% HCl)] *ad lib* A 12-hr light/dark cycle was used (light from 6 00 *a m* to 6 00 *p m*) Female rats (22 29 wk old, 240-315 g) were treated as indicated in the section *whole embryo culture* (WEC) Three male and three pregnant female rats were injected ip with 100 mg Aroclor 1254/kg body weight (Alltech, Deerfield) in olive oil, six and three days prior to hepatocyte isolation Three male and three pregnant female rats that were not injected with Aroclor 1254, served as controls Hepatocytes were isolated by the perfusion method as described previously (Piersma *et al*, 1991), except that for the perfusion Hank's balanced salt solution was used instead of Ham's F10 Maternal hepatocytes were isolated on day 10 of gestation The viability of the hepatocytes isolated, as assayed with trypan blue exclusion, was 88±3% Hepatocytes were suspended in supplemented William's medium E (Flow Laboratories, Irvine, Scotland) (Bechter *et al*, 1989) with 10% fetal calf serum (FCS) (Flow Laboratories, Irvine, Scotland) at a concentration of 8×10^5 cells/ml Cells were seeded on rat-tail collagen (Boehringer, Mannheim, Germany) coated petri dishes (10 cm ø) (Nunc, Roskilde, Denmark), 10 ml/plate and incubated at 37 °C with 5% CO_2 in air and with 100% humidity After 4 hr the medium was replaced by supplemented Williams' medium E without FCS, to which cyclophosphamide (Endoxan, purity>97%,

ASTA-Pharma, Bielefeld, Germany) was added (0, 10, 30, 100, 300 and 1000 μM) After 0, 1, 2, 4, 8 and 18 hr plates were taken (one for each concentration), the medium was sterile filtered (0 2 μm, Schleicher & Schuell, Dassel, Germany) and aliquots were frozen in fluid N2 and stored at -80 °C

Whole embryo culture

Human serum used in WEC was prepared as follows Blood was taken from adult volunteers (male and female) between 20 and 50 yr of age The blood was defibrinized during donation by shaking with glass pearls in a glass bottle The defibrinized blood was centrifuged immediately, the serum was heat-inactivated (30 min, 56 °C) and D-glucose was added (720 mg/l) After testing each individual serum for compatibility with WEC, the serum of two to five individuals was pooled and stored at -20 °C Female random bred Cpb WU (Wistar) rats (11-26 wk old, 200-300 g) were housed in pairs or three together in cages and were allowed access to food (MRH TM pellets, Hope Farms B V , Woerden) and water (tap water) *ad lib* A 12 hr light/dark cycle was used (lights from 12 00 *a m* to 12 00 *p m*) On day 0, females willing to mate were brought together with males (1 1) from 9 00 *a m* to 12 00 *a m* Embryos were explanted between 1 30 *p m* and 3 30 *p m* on day 10 Only embryos with four to eight somites were used for the culture The embryos were cultured separately for 24 hr in 25 ml glass bottles containing 2 ml medium at 37 5 °C and rotated at 30 rpm The culture medium consisted of 1 2 ml human serum and 0 8 ml supplemented Williams' medium E in which hepatocytes had been cultured The culture bottles were gassed with a mixture of O2, N2 and CO2 (5 90 5) After 2 and 18 hr of culture the bottles were gassed with a mixture of O2, N2 and CO2 (20 75 5) After culturing, the morphology of the embryos was evaluated as described by Brown and Fabro (1981) All dysmorphogenic features were recorded

Total cytochrome P450 content

Frozen-thawed hepatocytes were dissolved in 0 1 M phosphate buffer (pH 7 4) containing 23% glycerol and 0 1% Triton-X-100 The suspension was sonicated twice for 15 sec and hepatocyte cytochrome P450 was assayed by its carbon monoxide difference spectrum after reduction with sodium dithionite according to the procedure of Omura and Sato (1964) Protein content was assayed with Coomassie Brilliant Blue G (Sigma, St Louis, MO, USA) according to Bradford (1965) All samples were analysed in triplicate

Analysis of cyclophosphamide concentrations

Cyclophosphamide concentrations in the hepatocyte media were analysed with gas chromatography according to the method described for cyclophosphamide analysis of air samples, wipe samples and gloves by Sessink *et al* (1992) After identification of cyclophosphamide and isophosphamide (internal standard, Holoxan, purity>97%, ASTA-Pharma, Bielefeld, Germany) peaks by mass spectrometry (Sessink *et al* , 1992) routine analyses were performed with the same gas chromatographic method, but with a thermo ion-specific detector instead All samples were analysed in duplicate

Mutagenicity assay

For testing the mutagenicity of the hepatocyte media after incubation with cyclophosphamide, 0 4 ml of the sterile samples were added per plate and assayed with the *Salmo-*

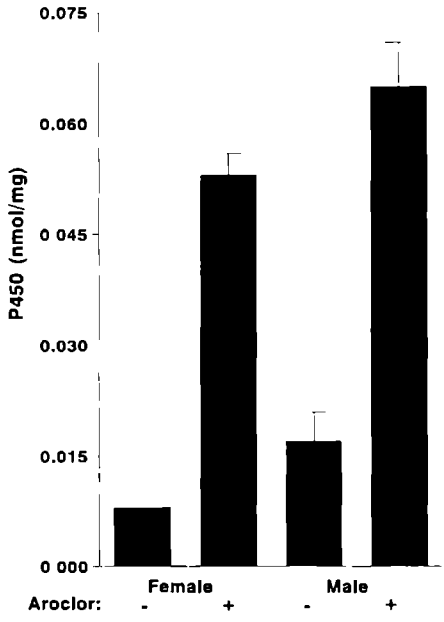

Fig 4 1 Total cytochrome P450 content of rat hepatocytes as determined by its carbon monoxide difference spectrum (nmol/mg cellular protein) Sex and Aroclor 1254 pretreatment as indicated Bars and error bars represent mean and SEM, respectively

nella typhimurium strain TA1535, according to Maron and Ames (1983) All samples were assayed in triplicate

Statistical and other calculations

Differences between groups were tested with Student's *t*-test The Bonferroni correction was used to correct for multiple comparisons with a nominal α of 0 05, differences were regarded as statistically significant at $P<0\ 05/k$ where k is the number of comparisons made The least square means of total morphological scores (TMS) and the number of dysmorphogenic features/conceptus were estimated by analysis of covariance, taking the number of somite pairs at the beginning of culture as covariant Pseudo-first-order rate constants of cyclophosphamide elimination from the hepatocyte media were calculated by a linear regression model in which the natural logarithm of the ratio of the concentration and the initial cyclophosphamide concentration was a function of the incubation time elapsed ($\ln([\text{cyclophosphamide}]/[\text{cyclophosphamide}]_{\text{initial}}) = k' \cdot \text{time}$) All calculations were performed with SAS statistical software v 6 06 on a Digital VAX 6000-410 computer

Results

Total P450 content

Total cytochrome P450 content after Aroclor 1254 pretreatment was increased 3 8-fold and 6 6-fold in male and female rats, respectively As a result of this the difference in total cytochrome P450 content between the sexes was greatly reduced after Aroclor 1254 pretreatment and it was no longer statistically significant (Fig 4 1)

Cyclophosphamide elimination

Hepatocytes isolated from Aroclor 1254 pretreated males eliminated cyclophosphamide from the medium approximately four times more rapidly than hepatocytes isolated from untreated males or Aroclor 1254 pretreated or un-

Sex	Aroclor 1254	k' (s^{-1})
Male	+	38 4 (1 2) x 10^{-6} *
Male	-	10 4 (1 2) x 10^{-6}
Female	+	9 5 (0 6) x 10^{-6}
Female	-	7 5 (2 8) x 10^{-6}

Table 4.1 Pseudo-first-order rate constants (k') for cyclophosphamide elimination from media in which hepatocytes were incubated in monolayer culture Sex and pretreatment with Aroclor 1254 of rats that provided the hepatocytes as indicated Each value was calculated on the basis of data from three different incubations with five different concentrations of cyclophosphamide (10-1000 μM) 'Values shown are means and SEM * = $P<0.0001$ in Students t-test when compared with each of the other values

treated females (Table 4.1, Fig. 4.2) There was no significant difference in cyclophosphamide elimination between the three latter groups.

Embryotoxicity and mutagenicity

When hepatocytes isolated from Aroclor 1254 pretreated male rats were cultured in the presence of 300 μM cyclophosphamide, within 1 hr, sufficient embryotoxic metabolites were produced to reduce the TMS and increase the number of dysmorphogenic features of conceptuses in a statistically significant way. Maximum embryotoxicity occurred after 4 hr. Thereafter embryotoxicity diminished (Fig. 4.3d). When hepatocytes were isolated from untreated male rats or from untreated or Aroclor 1254 pretreated female rats and were incubated in the presence of 300 μM cyclophosph-amide, only a small increase of embryotoxicity during the hepatocyte culture could be observed when embryos were cultured in these hepatocyte conditioned media. A statistically significant difference was only observed for the increase in the number of dysmorphogenic features in conceptuses cultured with the medium conditioned by untreated female rat hepatocytes (Fig. 4.3a-c). When hepatocytes were cultured in the presence of 1000 μM cyclophosphamide, embryotoxicity, as expressed by a reduced TMS and an increase of the number of dysmorphogenic features, could be observed in all experimental groups. The difference between groups under these circumstances was that the hepatocytes isolated from Aroclor 1254 pretreated males produced the embryotoxic metabolites more quickly than did the hepatocytes isolated from other rats. Because of the larger quantity of cyclophosphamide added to the medium, no diminishing of embryotoxicity could be observed (results not shown)

Similar patterns could be observed when media were assayed for mutagenicity. After incubation in the presence of 300 μM cyclophosphamide, hepatocytes isolated from untreated female rats produced no statistically significant level of mutagenic metabolites, as assayed with the Salmonella mutagenicity test. Hepatocytes isolated from Aroclor 1254 pretreated females or untreated males produced sufficient mutagenic metabolites to evoke a significant increase in

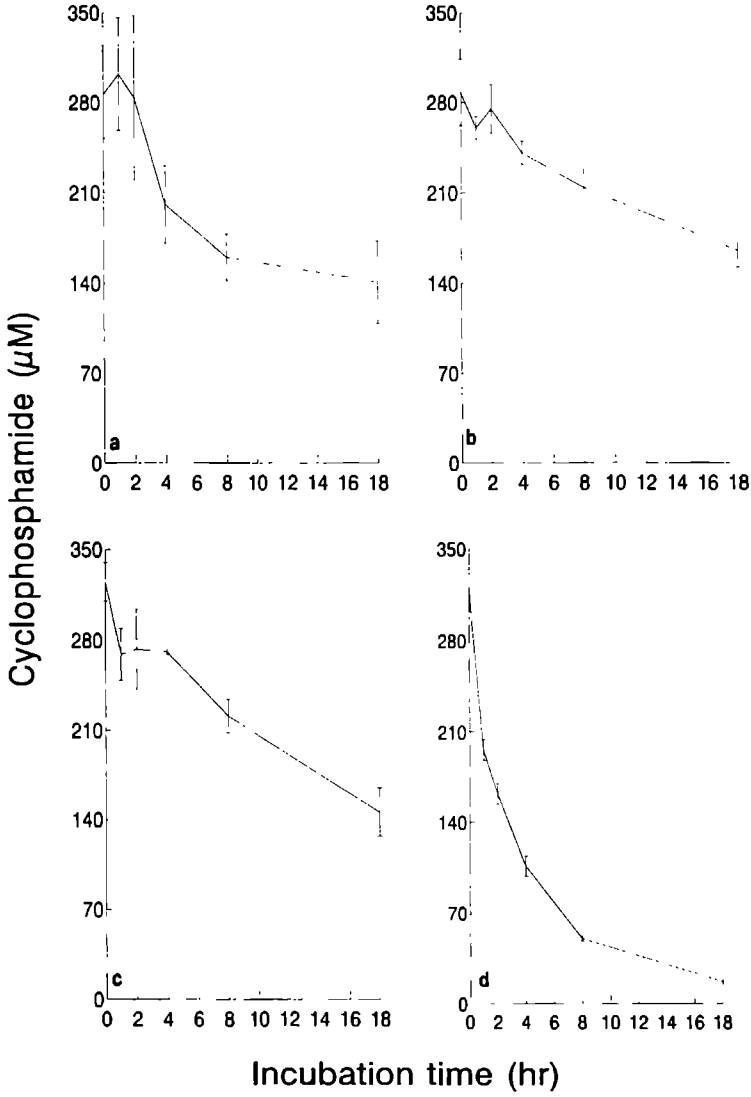

Fig. 4.2 Cyclophosphamide concentration against time curves for media in which hepatocytes were incubated in monolayer culture. Hepatocytes were derived from untreated pregnant female (a), Aroclor 1254 pretreatedpregnant female (b), untreated male (c) and Aroclor 1254 pretreated male rats (d). Values shown are means ± SEM. Each point consists of duplicate measurements of three samples from three different incubations.

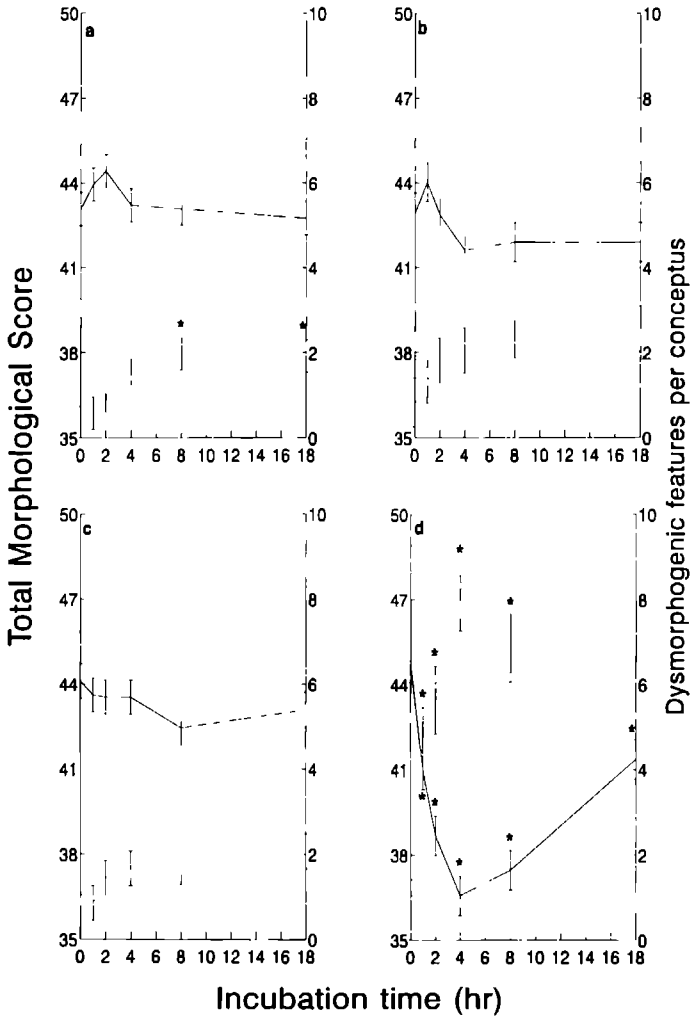

Fig. 4.3 Embryotoxicity of media in which hepatocytes were incubated in monolayer culture in the presence of 300 μM cyclophosphamide. Hepatocytes were derived from untreated pregnant female (a), Aroclor 1254 pretreated pregnant female (b), untreated male (c) and Aroclor 1254 pretreated male rats (d). Embryotoxicity is expressed as TMS (left axis, ___) and number of dysmorphogenic features per embryo (right axis, ----). Values shown are least square means ± SEM for nine embryos cultured in media from three different hepatocyte incubations. * = $P<0.008$ in Student's *t*-test when compared with samples from incubation time = 0.

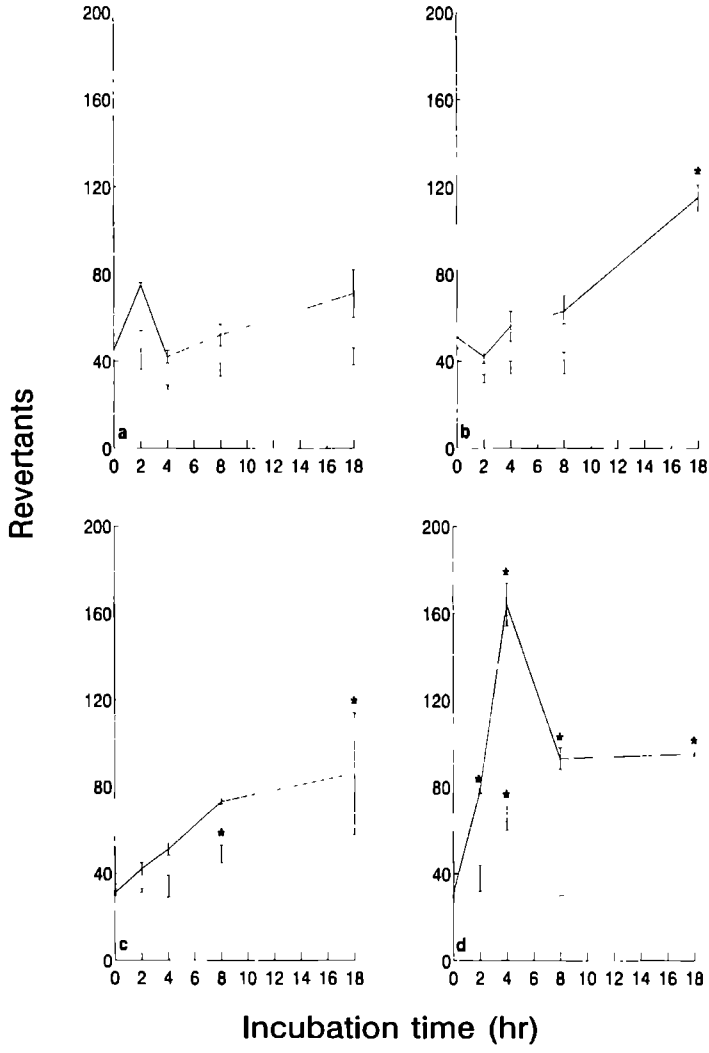

Fig. 4.4 Mutagenicity of media in which hepatocytes were incubated in monolayer culture in the presence of 300 μM cyclophosphamide (___) or no cyclophosphamide (----). Hepatocytes were derived from untreated pregnant female (a), Aroclor 1254 pretreated pregnant female (b), untreated male (c) and Aroclor 1254 pretreated male rats (d). Values shown are means ± SEM of revertants per plate for triplicate assays in the Salmonella mutagenicity test of media from three different hepatocyte incubations. * = $P<0.01$ in Student's *t*-test when compared with samples from incubation time =0.

bacterial mutants only after 18 hr of hepatocyte culture Hepatocytes isolated from Aroclor 1254 pretreated males bioactivated cyclophosphamide quickly enough to increase the mutagenicity of the medium within 2 hr in a statistically significant way In the latter group, the number of revertants was highest after 4 hr of hepatocyte culture However, since media in which hepatocytes from Aroclor 1254 pretreated males were cultured without cyclophosphamide also showed a significant increase in mutagenicity after 4 hr, this peak might be biased for some unknown reason (Fig 4 4)

Discussion

Our results show that the bioactivation of cyclophosphamide can be greatly enhanced in male rats after Aroclor 1254 pretreatment, while this P450-enzyme inducer has only little effect on the bioactivation of cyclophosphamide in pregnant female rats A similar sex difference in the inductive capacity of Aroclor 1254 on the bioactivation of a xenobiotic has been reported by Juchau *et al* (1985), who showed that the production of hydroxylated metabolites of 2-acetylaminofluorene was much more enhanced by Aroclor 1254 in male livers than in maternal livers When rat embryos were cultured in the presence of 2-acetylaminofluorene, crude microsomal fraction (S9) prepared from Aroclor 1254 pretreated male livers elicited many malformations in the embryos, whereas addition of S9 prepared from Aroclor 1254 pretreated maternal livers had no embryotoxic effect at all Cytochrome P450 iso-enzymes CYP1A1 (for terminology see Nebert *et al*, 1991), participating in the hydroxylation of 2-acetylaminofluorene, and CYP2B1, the main P450 iso-enzyme bioactivating cyclophosphamide after phenobarbital induction (Clarke and Waxman, 1989, Giachelli and Omiecinski, 1985), are known to be induced by Aroclor 1254 (Ryan *et al*, 1978) The distribution of these iso-enzymes is not sex specific, although there are sex differences in the levels of expression (Waxman *et al*, 1985, Clarke and Waxman, 1989) The observed sex differences in inductive capacity of Aroclor 1254 remain therefore partly unexplained One factor that might have contributed to the observed effects, is the difference in age between the male rats and the pregnant female rats used Indeed it has been reported that the phenobarbital responsiveness of CYP2B1 mRNA expression is highest at the 46th day of post-natal development of the rat and is decreased to 40% of the peak level at the 65th day Whether there is a further decrease of CYP2B1 inducibility thereafter is not known

Another element that might have attributed to the observed results is pregnancy It is known that the cytochrome P450 content of rat maternal liver decreases during pregnancy (Brown *et al*, 1986, Nakajima *et al*, 1992) Indeed it has been shown that two iso-enzymes, involved in cyclophosphamide bioactivation, namely CYP2C6 and CYP2B1 (Clarke and Waxman, 1989), are present at a

somewhat lower level in pregnant female rats on day 10 of gestation than in non-pregnant female rats (Nakajima *et al*, 1992) Whether these slightly lower levels of expression during pregnancy render these iso-enzymes much less susceptible to Aroclor 1254 induction is not known

An interesting feature of the sequential culture model, as applied here, is the possibility to follow the appearance and disappearance of embryotoxic metabolites time-dependently Our results show that embryotoxic and mutagenic metabolites appear simultaneously when Aroclor 1254 pretreated male rat hepatocytes are incubated in the presence of 300 μM cyclophosphamide After 4 hr of incubation the embryotoxicity of the medium decreases significantly, while the mutagenicity of the medium remains high This might be explained as follows first cyclophosphamide is bioactivated cytochrome P450-dependently to 4-hydroxycyclophosphamide The tautomer of 4-hydroxycyclophosphamide, aldophosphamide, degrades spontaneously in aqueous solution, which results in the formation of acrolein and phosphoramide mustard (Slott and Hales, 1988) Acrolein is very embryotoxic, but not mutagenic (Ellenberger and Mohn, 1977, Hales, 1982) and is detoxified by a reaction with glutathione (Ohno and Ormstad, 1985) This reaction is probably enhanced, since glutathione transferases are also induced by Aroclor 1254 Phosphoramide mustard, on the other hand, is both a mutagen and a dysmorphogen If phosphoramide mustard is more persistent in the medium, this could explain the differential detoxification rates as observed for the mutagenicity and the embryotoxicity of the hepatocyte media

Production Of Cyclophosphamide Metabolites By Primary Hepatocyte Cultures From Male And Pregnant Rats Effect Of Aroclor 1254 Pretreatment

5

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Toxicology in vitro 9:151-156

Abstract In a previous paper (VanAerts *et al.*, *Toxicology in Vitro* 1993, **7**, 769-775) we have shown that the embryotoxicity of cyclophosphamide was greatly enhanced when cyclophosphamide had been added to a primary hepatocyte culture derived from Aroclor 1254-pretreated male rats (MA) and the medium from this culture was added to a post-implantation rat embryo culture. However, when medium from hepatocytes that had been derived from untreated male rats (MC), untreated pregnant rats (PC) or Aroclor 1254-pretreated pregnant rats (PA) was used embryotoxicity was low. We now present data on the concentrations of 4-ketocyclophosphamide, carboxyphosphamide and nornitrogen mustard in the primary hepatocyte culture media. 4-Ketocyclophosphamide was absent in media from PC or PA and present in low concentrations in media from MC and MA. Carboxyphosphamide and nornitrogen mustard were present in all media, but their concentrations were several times higher in medium from MA than in media from other sources. This indicates that Aroclor 1254 pretreatment enhances cyclophosphamide metabolism much more in male rats than in pregnant rats It is suggested that both a greater inducibility of CYP2B1 and augmented stabilization of 4-hydroxycyclophosphamide by glutathione conjugation may be processes that contribute to the observed differences in metabolite concentrations.

Introduction

The culture of post-implantation rodent embryos has become a well-established tool for investigating the embryotoxicity of chemical substances. However, many substances need bioactivation before they exert any embryotoxic effects. Therefore this model has been extended by introducing external biotransforming systems. One of the approaches used for this purpose is the sequential culture model (Bechter *et al.*, 1989, VanAerts *et al.*, 1993b). In this model a prodysmorphogen is added to a primary rat hepatocyte culture. The medium derived from this culture is used for the culture of post-implantation rat embryos to

assess the embryotoxicity of the metabolites formed. In order to enhance the biotransforming capacity of the hepatocytes used, rats are often pretreated with Aroclor 1254, a commercial mixture of polychlorinated biphenyls. Routinely male rats are used for this purpose. However, using pregnant rats would be a more physiological approach to investigate the embryotoxicity of prodysmorphogens. We designed an experiment in which hepatocytes were derived from four different sources: untreated male rats (MC), Aroclor 1254-pretreated male rats (MA), untreated pregnant rats (PC) and Aroclor 1254-pretreated pregnant rats (PA) (VanAerts *et al.*, 1993b). It was shown that the embryotoxicity was much greater when medium from cyclophosphamide-exposed MA hepatocyte cultures was used than when media from hepatocytes from other sources were tested. Lack of enhancement of cyclophosphamide teratogenicity by Aroclor 1254 pretreatment was also reported by Welsch (1985), who used mice and assessed teratogenicity *in vivo*. This author even found a diminished teratogenicity of cyclophosphamide after Aroclor 1254 pretreatment.

We now extend our studies by presenting data on the levels of carboxyphosphamide, 4-ketocyclophosphamide and normitrogen mustard found in the media in which hepatocytes had been incubated with cyclophosphamide for different time intervals. The media that have been analysed, are the same as those that have been used for cyclophosphamide analysis, rat embryo culture and mutagenicity testing for which the data have been published in a previous paper (VanAerts *et al.* 1993b).

Materials and methods

Chemicals

Cyclophosphamide, trophosphamide, carboxyphosphamide and 4-ketocyclophosphamide were gifts from Asta-Medica (Frankfurt am Main, Germany) Normitrogen mustard was purchased from Aldrich Chemical Co (Milwaukee, WI, USA) Benzenesulfonylchloride was obtained from Janssen Chimica (Beerse, Belgium) *N,N*-dimethylformamide dimethyl acetal was purchased from Chrompack (Middelburg, The Netherlands) All other chemicals were of the highest purity obtainable

Experimental design

A detailed description of the experimental design is given in VanAerts *et al* (1993b) Briefly, hepatocytes were isolated from MC, MA, PC and PA by a perfusion method and incubated in the presence of 300 μM cyclophosphamide in a monolayer culture (8×10^6 cells/10 ml dish) In each group three rats were used Media samples were taken at different time intervals (0, 1, 2, 4, 8 and 18 hr) These media were used for embryo culture, mutagenicity testing and analyses of cyclophosphamide and its metabolites carboxyphosphamide, 4-ketocyclophosphamide and normitrogen mustard

Metabolite analyses

All media samples (three per group at each time interval) were analyzed in duplicate

Analyses of metabolites were adapted from the methods developed by Sessink *et al* (in press) Briefly, for measurement of carboxyphosphamide and 4-ketocyclophosphamide samples were diluted with water and Tris buffer (pH 8 0) Excess of cyclophosphamide was removed by diethylether extraction to avoid interference during the analysis The ether layers were discarded and 6 M HCl was added The mixture was extracted with ethylacetate. The ethylacetate layers were dried under nitrogen at 40°C The residue was dissolved in toluene, containing *N,N*-dimethylformamide dimethyl acetal, and derivatized at 115°C After derivatization the samples were dried under nitrogen at 50°C. The residues were dissolved in toluene, containing 1 μg trophosphamide (internal standard) The derivatives were analysed by gas chromatography with nitrogen phosphorus detection

For measurement of nornitrogen mustard the samples were diluted with water and Tris buffer (pH 8 0) Diethylether containing benzenesulphonylchloride was added and the samples were shaken for 90 min The ether layers were removed and dried under nitrogen at 30°C Finally, the residue was dissolved in toluene The nornitrogen derivative was analysed by gas chromatography with mass spectrometric detection

Statistical analysis

Rates of metabolite production were calculated by linear regression analysis Differences in metabolite levels or rates of metabolite production were tested with Student's *t*-test Since six comparisons were made in each test a difference was only regarded statistically significant at $P<0\ 01$. When metabolite levels were below the detection limit, 0 was used as a value for the calculations

Results

The concentrations of carboxyphosphamide, 4-ketocyclophosphamide and nornitrogen mustard present in the media after incubation with hepatocytes are depicted in Fig 5.1. 4-Ketocyclophosphamide was not detected in media from PC or PA. However, 4-ketocyclophosphamide was formed at a rate of $0\ 30\pm0.15$ nmol/hr·10^6 cells (mean ± standard deviation) by MC and this rate increased significantly to 1.7 ± 0.4 nmol/hr·10^6 cells when the male rats had been Aroclor 1254 pretreated Carboxyphosphamide was detected in the media from all experimental groups. In PC the rate of carboxyphosphamide formation was 1.5 ± 0.3 nmol/hr·10^6 cells. This rate was not significantly increased in PA ($2\ 6\pm1.6$ nmol/hr·10^6 cells). In MC the rate of carboxyphosphamide formation was between those observed in PC and in PA ($2\ 2\pm1.1$ nmol/hr·10^6 cells). This rate increased significantly to $6.6\pm0\ 7$ nmol/hr·10^6 cells when male rats were pretreated with Aroclor 1254. Nornitrogen mustard was also detected in media from all experimental groups. In PC the rate of nornitrogen mustard formation was $0.20\pm0\ 04$ nmol/hr·10^6 cells This rate was not significantly increased in PA ($0\ 50\pm0\ 23$ nmol/hr·10^6 cells) In MC the rate of nornitrogen mustard formation was between those observed in PC and PA ($0.35\pm0\ 04$ nmol/hr·10^6 cells) This rate increased to $1\ 9\pm0.8$ nmol/hr·10^6 cells when male rats were pretreated with

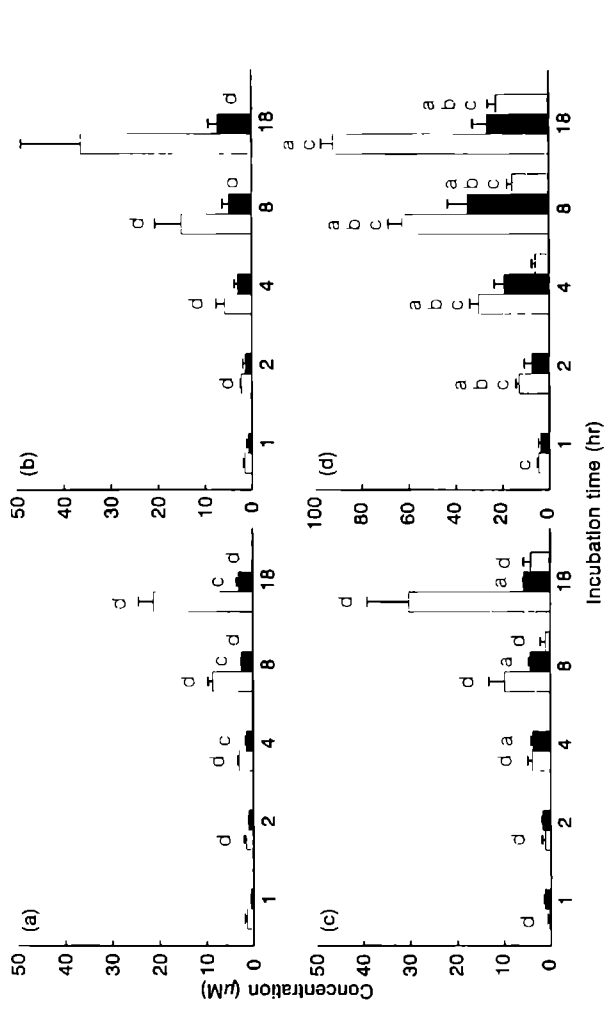

Fig 5.1 Carboxyphosphamide (white), normitrogen mustard (black) and 4-ketocyclophosphamide (shaded) concentrations in media from primary hepatocyte cultures to which 300 μM cyclophosphamide had been added. Hepatocytes were derived from a) untreated pregnant rats, b) Aroclor 1254-pretreated pregnant rats, c) untreated male rats and d) Aroclor 1254-pretreated male rats. Values shown are means of three separate incubations. Error bars represent the SEM. Characters above bars indicate a significant difference in Student's *t*-test ($P<0.01$) in comparison with the corresponding metabolite level in the panel indicated by the character

Aroclor 1254. However because of the larger variation of nornitrogen mustard concentrations in this group and non-linearity of the concentration against time curve, this increase did not reach a statistical level of significance ($0.01<P<0.05$). On the other hand in hepatocytes derived from untreated rats the nornitrogen formation was significantly higher in those from males than in those from pregnant females. The much higher levels of metabolites in media from MA is therefore partly due to the slightly higher basic rate of formation of metabolites by male-derived hepatocytes than in hepatocytes derived from pregnant females. The major cause of the much higher levels of metabolites, however, is a larger effect of Aroclor 1254 pretreatment on male hepatocytes.

Discussion

Carboxyphosphamide and 4-ketocyclophosphamide are stable non-toxic oxidative metabolites of aldophosphamide and 4-hydroxycyclophosphamide, respectively. They are formed by NAD(P)-linked cytosolic aldehyde dehydrogenases (ALDHs) (Cox *et al.*, 1976; Domeyer and Sladek, 1980) and probably an aldehyde oxidase (Hohorst *et al.*, 1971), respectively (Fig. 5.2). Our results confirm that 4-ketocyclophosphamide is only a minor metabolite formed by rat hepatocytes (Sladek, 1988). We could not detect 4-ketocyclophosphamide in the media from PC or PA.

Treatment with Aroclor 1254 increased the formation of carboxyphosphamide. Theoretically this could be due to induction of ALDHs (Lindahl, 1992; Deitrich *et al.*, 1978). However, the relative abundance of ALDHs in liver tissue and the low K_m of some of them for the oxidation of aldophosphamide makes it less likely that even in an uninduced state the aldophosphamide oxidation capacity of hepatic ALDHs is limiting (Manthey *et al.*, 1990). Therefore higher levels of carboxyphosphamide are most probably not the result of induction of subsequent metabolism of aldophosphamide, but rather reflect higher steady state levels of aldophosphamide.

Phosphoramide mustard is formed spontaneously from aldophosphamide by ß-elimination of acrolein (Sladek, 1988). In an aqueous solution, however, phosphoramide mustard will spontaneously degrade to nornitrogen mustard (Jardine *et al.*, 1978). Therefore nornitrogen mustard levels measured in the media will reflect the levels of activated cyclophosphamide, (*i.e.* 4-hydroxycyclophosphamide and its tautomere aldophosphamide), as well.

Higher levels of activated cyclophosphamide may be the result of a higher rate of formation or of a greater persistence of activated cyclophosphamide in the medium. The formation of activated cyclophosphamide in hepatocytes is primarily mediated by P450 isozymes (Sladek, 1988; Clarke and Waxman, 1989; Kanekal and Kehrer, 1993). After Aroclor 1254 treatment, CYP2B1 is the princi-

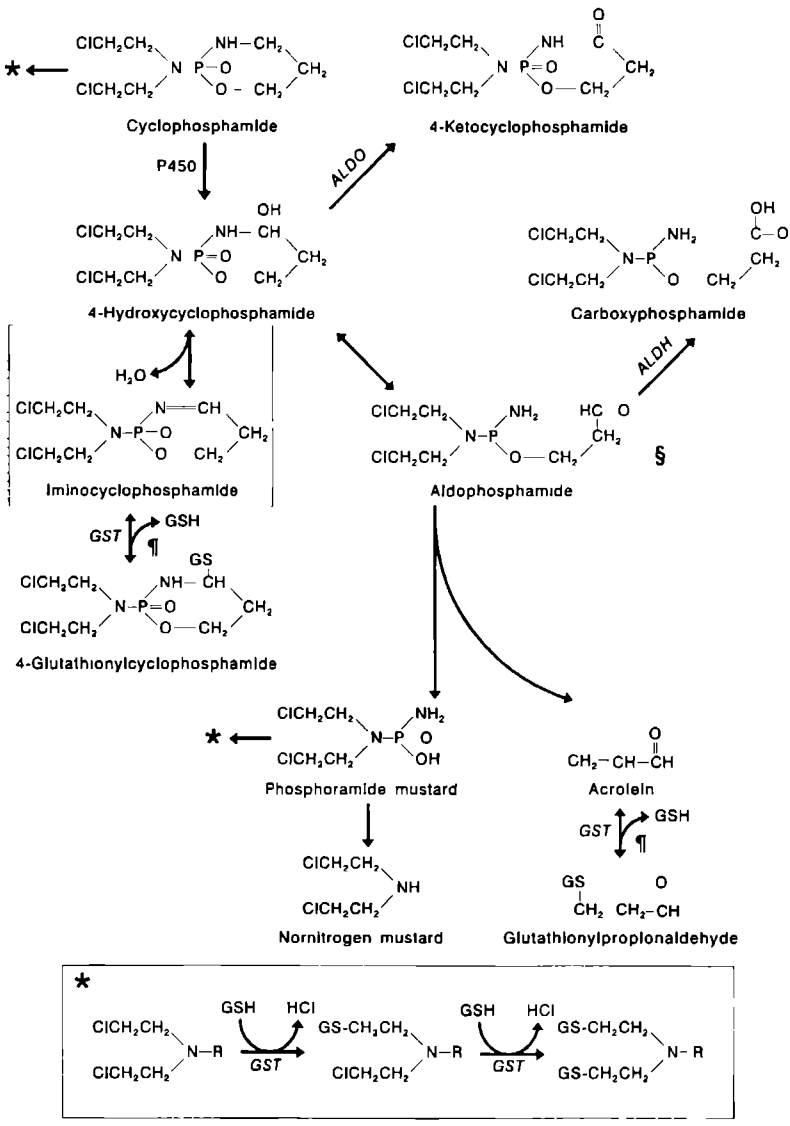

Fig. 5.2 Schematic representation of the biotransformation of cyclophosphamide ★ Cyclophosphamide and phosphoramide mustard have been shown to be metabolized to their mono- and diglutathionylconjugates by glutathione-*S*-transferase (*GST*) by direct displacement of the chloride atoms and through aziridinium intermediates, respectively, as represented in the inset § Transport forms of activated cyclophosphamide are within the dashed frame ¶ These metabolites may also react spontaneously with other sulfhydryl compounds to form their respective mercapto derivatives *ALDH* = aldehyde dehydrogenase *ALDO* = aldehyde oxidase P450 = cytochrome P450 mono-oxygenase GSH = glutathione

pal mono-oxygenase hydroxylating cyclophosphamide (Borlakoglu *et al*, 1993, Parkinson *et al*, 1983a,b, Ryan *et al*, 1979), However, to our knowledge a sex difference in CYP2B1 inducibility has never been reported

Prolonged persistence of activated cyclophosphamide in the medium may have been caused by glutathione conjugation of 4-hydroxycyclophosphamide (Pallante *et al*, 1986, Lee, 1991, Fenselau *et al*, 1982) The conjugate, 4-glutathionylcyclophosphamide, is of moderate stability in an aqueous solution (Draeger *et al*, 1976, Peter *et al*, 1976) When it deconjugates, activated cyclophosphamide is formed once again and this process has therefore been referred to as 'delayed toxication' (Hohorst *et al*, 1976) Total GST activity, using 1-chloro-2,4-dinitrobenzene as a substrate, is about 1 5-fold higher in male rats than in female rats and GST activity can be induced by classical inducers, like 3-methylcholantrene, phenobarbital and Aroclor 1254 (Kaplowitz *et al*, 1975, Coecke *et al*, 1990, Borlakoglu *et al*, 1993) Gender-specific inducibility of GST subclasses has been reported for 3-methylcholantrene and phenobarbital, but not for Aroclor 1254 (Igarashi *et al*, 1987)

Gender may not have been the only factor causing the observed differences in our experiments The pregnant state of the females may have contributed as well It is known that pregnancy reduces P450 levels (Feuer, 1979, Neale and Parke, 1973) It has also been shown that the inducibility of ethylmorphine *N*-demethylase by phenobarbital, is reduced two-fold during pregnancy in rats (Guenthner and Mannering, 1977) However, CYP2B1 mRNA levels in pregnant rats were increased 40-fold after Aroclor 1254 treatment (Borlakoglu *et al*, 1993)

Thus, further research will be needed to determine if reduced inducibility of CYP2B1 by Aroclor 1254 treatment in pregnant rats, in comparison with male rats, or increased GST activity in Aroclor 1254 pretreated male rats may have caused the large difference in the formation of embryotoxic metabolites of cyclophosphamide as it was observed in our sequential model Furthermore a possible effect of pregnancy on these processes will have to be investigated as well

Our observations make it clear that the choice of the external bioactivating system used in the sequential culture model has a major influence on the outcome of the experiments when this model is used to study prodysmorphogens

Acknowledgements We gratefully acknowledge the assistance by P van den Broek and B van de Griendt in the analysis of the cyclophosphamide metabolites We also wish to thank P Sessink and R Bos for making the analytical procedures available to us

Summary And Conclusions

6

The capacity of postimplantation rat embryos to metabolize xenobiotics is limited. Therefore if the role of biotransformation in teratogenesis is investigated or when one wants to use whole embryo culture as a pre-screening tool and does not want to exclude the possible effects of metabolites, the postimplantation rat embryo culture model has to be extended with an external bioactivating system.

We therefore set out to combine the culture of rat embryos with the culture of intact rat hepatocytes. This work was started at the National Institute of Public Health and Environment in the Netherlands in collaboration with Piersma and co-workers (VanAerts *et al.*, 1989; Piersma *et al.*, 1991). In this original approach the hepatocytes were cultured in suspension in the serum in which the embryos were cultured as well. This model was designated as the *co-culture model.* It was shown that cyclophosphamide, which was used as a model compound, elicited embryotoxicity at a concentration of 30 μg/ml in the presence of hepatocytes, whereas in the absence of hepatocytes no embryotoxicity was observed up to a concentration of 1000 μg/ml.

Another approach to the combination of the culture of hepatocytes and the culture of rat embryos was made by Bechter *et al* (1989). They first cultured the hepatocytes in a monolayer culture. From this culture media samples were taken at various time intervals. These media were combined with serum, in which the embryos were cultured. This model was designated as the *sequential culture model.*

In chapter 3 a comparison between the co-culture model and the sequential culture model was made. The sensitivity of both models was comparable. However, both models have their advantages and disadvantages. In the co-culture

model the hepatocytes and the embryos are cultured simultaneously Instable metabolites are therefore able to elicit embryotoxic effects, since they have direct access to the embryos, once they are released by the hepatocytes However, in the sequential culture model the media in which the hepatocytes are incubated are stored and added later on to the embryo culture Therefore some loss of instable metabolites may occur in this system On the other hand in the co-culture model only an endpoint measurement is made That is, the sum of the effects of the formation of toxic metabolites and the subsequent detoxification of them is observed, since the hepatocytes are present throughout the incubation period However, in the sequential culture model it is possible to observe the temporal effects of bioactivation and detoxification, since medium samples of the hepatocytes culture are taken at different time intervals The most pragmatic approach is probably to start out with the co-culture model to get a general impression of the effect of metabolism of a certain compound, whereas the sequential culture model can be applied to get a more detailed view of the bioactivation and detoxification processes Another practical advantage of the sequential culture model is, that only a few hepatocytes isolations and incubations have to be performed in order to gather sufficient medium for all embryo cultures, whereas in the co-culture model for each embryo culture hepatocytes have to be isolated as well

It was noted that the sensitivity of the sequential culture model as observed by us was at least ten times lower than the sensitivity of this model as reported by Bechter *et al* (1989) A likely cause for this discrepancy was the nature of the hepatocytes used Bechter had used hepatocytes from Aroclor 1254 pretreated male rats, whereas we used hepatocytes from untreated pregnant females To investigate whether the observed differences in sensitivity were caused by the Aroclor 1254 pretreatment of the rats, or were caused by the sex difference, we designed an experiment in which both factors were varied (chapter 4 and 5) It was found that neither gender nor Aroclor 1254 pretreatment alone were sufficient to explain the differences between Bechter's and our results Instead it was found that only in male rats the biotransformation of cyclophosphamide was greatly enhanced by Aroclor 1254 pretreatment The principal cytochrome P450 mono-oxygenase responsible for cyclophosphamide activation after Aroclor 1254 pretreatment is CYP2B1 (Borlakoglu *et al* , 1993, Parkinson *et al* , 1983a, 1983b, Ryan *et al* , 1979) However, gender-specificity for the induction of this cytochrome has never been reported We speculated that instead of an increased formation an increased persistency of 'activated' cyclophosphamide may have occurred as a result of differences in the activity of GST's, which may have lead to differences in the degree in which 4-glutathionylcyclophosphamide was formed Nevertheless on the basis of the results presented in this thesis no firm conclusion can be drawn as to whether the increased biotransformation of cyclophosphamide by male hepatocytes after Aroclor 1254 pretreatment is

caused by increased formation of 'activated' cyclophosphamide due to a gender-specific difference in the rate of induction of CYP2B1 or that the differences in embryotoxicity reflected differences in the extent to which 'activated' cyclophosphamide was stabilised by 4-glutathionylcyclophosphamide formation. To answer this question further research will have to be conducted. Furthermore in our experiments pregnant females were used. The relatively small effect of Aroclor 1254 pretreatment in pregnant rats on the biotransformation of cyclophosphamide may be caused by gender-specific properties of the rats as well as by the pregnant state these rats were in. Future investigators should recognize both of these possibilities and design their experiments in such a way that differences in their observations can be delineated to either of these factors.

The studies presented in this thesis show that it is possible to combine the culture of hepatocytes with culture of rat embryos, both by co-culturing them in one bottle as well as by a sequential culture model. Our studies also clearly demonstrate that the choice of the source of the hepatocytes may have a major influence on the outcome of the experiments and should therefore carefully be considered.

PART II

INVESTIGATIONS ON THE POSSIBLE ROLE OF HOMOCYSTEINE IN THE AETIOLOGY OF NEURAL TUBE DEFECTS

Introduction

Neurulation

According to Adelmann (1966) the earliest significant contributions to the embryological study of neurulation - the formation of the neural tube - were made by Malpighi (1672). Despite the simple structure of microscopes in the early days he clearly saw and described the neural walls in the chicken embryo. Nevertheless Malpighi did not discover the true meaning of them, being the very anlagen of the central nervous system. Instead he interpreted them as walls 'guarding' the space between them within which the cerebral vesicles were formed by a 'plastic spirit' (Malpighi, 1697). Even one and a half century later a famous embryologist as Von Baer (1828) maintained Malpighi's interpretation that the cerebral vesicles were deposited within the lumen of the neural walls. It were Rusconi (1826) and Reichert (1840; 1842) who first appreciated the full significance of the neural walls .

Today neurulation is considered to occur in two phases: primary and secondary neurulation (Fig. 7.1). At the time the neural walls are visible primary neurulation has already proceeded half the way as it actually starts with the formation of the neural plate. Formation of the neural plate is initiated as early as the primitive streak stage. In the rat this is at gestational day 8.5, in humans approximately in the first half of week 3. During the primitive streak stage ectodermal cells of the epiblast migrate ventrally and laterally through the primitive streak to form the mesodermal layer, displace the hypoblastic endoderm into the extra-embryonic endoderm and form the definitive embryonic endoderm. This process is often referred to as gastrulation. Little is known of the process of neural induction - i.e. the formation of the neural plate - in mammals. However based

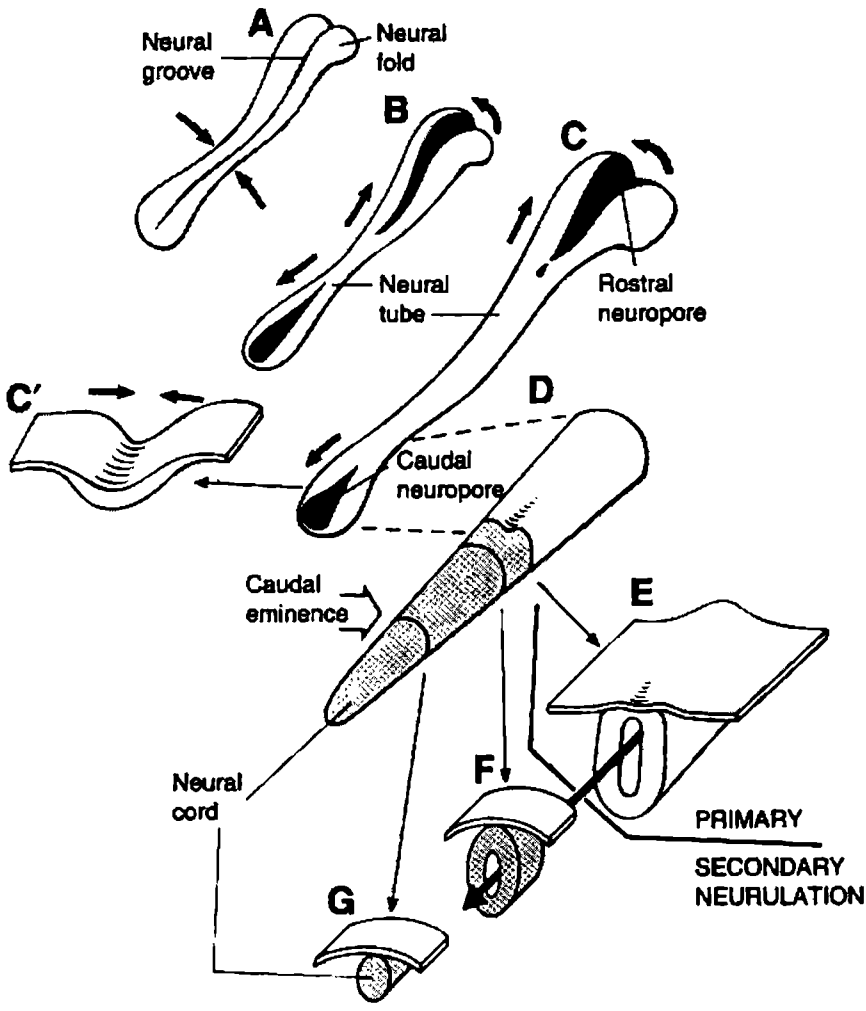

Figure 7.1 Formation of the neural tube in humans. The neural folds (A) begin to fuse (B) at about 22 days. At 24 and 26 daysthe rostral and caudal neuropores close (C). In D and E a slight pit indicates the position of the former caudal neuropore, beyond which the neural tube is formed by secondary neurulation (shaded). In G a solid cord has formed beneath the surface ectoderm. More rostrally (F) a lumen has appeared in the cord and is continuous with that formed during primary neurulation (arrow). Reprinted from O'Rahilly and Müller (1992) by permission of John-Wiley-Liss Inc. © 1992 John Wiley & Sons Inc.

on presumed parallels with amphibian embryos, it is generally believed that the neural plate is formed by induction of dorsal ectodermal cells of the epiblast by the underlying mesodermal and possibly endodermal cells, as well as by the notochord (Morris-Kay *et al* , 1994, Schoenwolf, 1994) The earliest neural plate consists only of tissue that will develop into the forebrain, midbrain and the most rostral part of the hindbrain The subsequent broadening and lengthening of the neural plate occurs in the rat between gestational day 8 5 and gestational day 9 5 and in humans between gestational day 16 and gestational day 19 Broadening of the neural plate is due to a change of epithelial structure from pseudostratified to cuboidal, the cell number remaining constant Cell division contributes only to the lengthening of the neural tube During this phase the neural groove appears and the neural plate gets the appearance of two neural folds which originally have a convex shape - i e the lateral edges are bent ventrally

At gestational day 9 5 in the rat - approximately gestational day 20 in humans - the lateral edges of the neural folds start to elevate Many processes contribute to the elevation and bending of the neural folds The following brief description is mainly based on studies on neurulation in the chicken (Schoenwolf, 1994), but most of the processes described are applicable to mammals as well (Morris-Kay *et al* , 1994, O'Rahilly and Muller, 1992, Stanisstreet, 1987) An important event is the change of cell shape of the neuroepithelial cells The lateral neuroepithelial cells increase in hight as they get a high columnar, spindle-shaped configuration and the neuroepithelial cells at the median hinge-point decrease in hight as they get a low columnar wedge shaped configuration The change and maintenance of cell shape and curvature of the neural folds is achieved by the action of microfilaments and microtubules Also apicobasal flattening and intercalation of surface ectodermal cells contribute to the bending of the neural folds Another extrinsic force - i e lying outside the neural tissue - is the expansion of the extracellular matrix of the underlying mesenchyme, which provides in this way a mechanical force pushing the neural tissue dorsally and medially This extracellular matrix could also have a regulatory influence on cell behaviour thereby affecting other events important for neurulation Intercalation of neuroepithelial cells in the transverse plane assists the narrowing and concomitant extension of the neural tube, whereas new cells that are deposited in the longitudinal direction contribute to the elongation of the neural tube As a result of the elevation and bending of the neural folds the apices of the neural folds finally appose along the dorsal midline where the neural tube starts to close by adherence and fusion of the apices For this the formation of an adhesive glycoconjugate coat on the cell surface is needed Also protrusive activity of the cells in the neural fold transition zone is involved At gestational day 10 3 in the rat (gestational day 22 in humans) fusion starts at the cervical region at the level of somite 1-3 From this point fusion proceeds rostrally and caudally Previously this fusion initiation point was considered to be the only one and fusion was

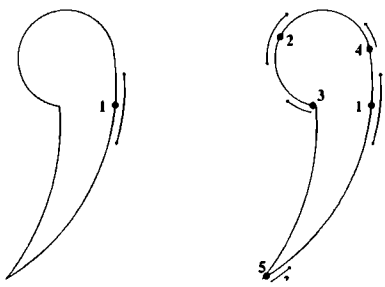

Fig. 7.2 Schematic drawing illustrating the multi-closure model vs the single-closure model (also referred to as the 'zipper model') of primary neurulation in mammals After Golden and Chernoff (1993) Reprinted from Golden and Chernoff (1993), by permission of John-Wiley-Inc © 1993 John Wiley & Sons Inc.

thought to proceed until the most rostral part - the rostral neuropore - and the most caudal part - the caudal neuropore - was reached. However Golden and Chernoff (1983; 1993) showed in three mouse strains that there were at least four different closure initiation sites, the first being the cervical one just mentioned. Closure II takes place at the prosencephalic-mesencephalic border and also proceeds bidirectional. Closure III is unidirectional, beginning adjacent to the stomodeum and proceeding caudally to meet closure II. Finally closure IV takes place over the rhombencephalon where it meets closure II to complete rostral neural tube closure (Fig. 7.2). Also Sakai (1989) observes multiple closure sites in the mouse, however concludes differences may exist between different strains. Rostral neural tube closure is complete at gestational day 10.8 in the rat and at gestational day 24 in humans. Golden and Chernoff (1993) reviewed the literature and found tentative evidence that multi-closure of neural tube formation occurs also in the hamster, the rat, the rabbit, the pig and in humans. O'Rahilly and Müller (1994) recognize that there is at least a second rostral closure initiation site in the human, which is equivalent to closure II as proposed by Golden and Chernoff (1993). Van Allen *et al.* (1993) studied the spatial distribution of neural tube defects in humans and concluded that a multi-closure model as proposed by Golden and Chernoff (1993) provided the best explanation for their observations. They even suggested that the most caudal neural tube defects might be related to the failure of a fifth closure event at the level of L2 to S2. Neural tube closure is not homogenous at all sites. Especially the rhombencephalic closure (closure IV) is unlike the others as it does not occur by fusion of the neural folds, but rather by elongation of a membrane which eventually covers the rhombencephalon.

With the closure of the caudal neuropore (gestational day 11.2 in the rat, gestational day 26 in humans) primary neurulation has completed and secondary neurulation can start. Caudally of the previous caudal neuropore the tail bud (caudal eminence) has formed. It comprises of undifferentiated mesenchymal cells and represents the remains of the primitive streak (Griffith *et al.*, 1992). This seemingly homogenous tissue gives rise to various tissues including the

hind tail gut, somites and their derivatives and neural crest cells. During secondary neurulation peripheral cells of the medullary cord arrange radially and differentiate from a mesenchymal to an epithelial form (the neural cord). The lumen of the primary neural tube proceeds caudally into the neural cord, thus giving rise to the secondary neural tube. In humans the process of secondary neurulation is complete at the sixth week of gestation. At the end of the embryonic period the neural cord still reaches till the end of the vertebral column, but during the fetal period it regresses to sacral and then lumbar levels.

Neural tube defects

Neural tube defects (NTDs) are some of the most distressing congenital malformations that occur when the process of neural tube closure, which takes place in humans in the fourth week post conception, fails. The most common forms of NTDs are spina bifida and anencephaly. Their prevalence at birth varies between countries and socioeconomic and ethnic groups. In Finland the prevalence frequency is only 1 in 2500. In Mexico it is 1 in every 300 births. Even higher rates have been reported in the past, i.e. 12 per 1000 births in the coal-mining valleys of South-Wales. On average the prevalence frequency is 1 in 500 (International Clearinghouse, 1991; Elwood and Elwood, 1980; Carter, 1974; Laurence *et al.*, 1967). In the Netherlands the prevalence of NTDs is 1 in 700 (EUROCAT Working Group, 1991). In these figures the affected embryos and foetuses that are spontaneously aborted are usually not included. Of the foetuses that are spontaneously aborted the frequency of NTDs has been estimated to be ten-fold higher as compared to in term births (Creasy and Alberman, 1976; Byrne and Warburton, 1986). Shiota (1993) estimated the prenatal mortality rate of NTD-affected embryos to be 98.4%, and most (93%) of the embryos do not survive the embryonic period proper. Thus the prevalence of NTDs at the end of the neurulation is ten to fifty times as high as observed at birth.

Based on their aetiology and appearance NTDs can be subdivided in two groups: the so-called 'open' and the 'closed' NTDs. In open NTDs the defect has arisen due to non-closure of the neural tube. Also rupture of the neural tube after a transient closure has been suggested as a rare cause for open NTDs (Hook, 1992). The subsequent development of surrounding tissues will be disturbed, which will result in exposure of the neural tissue at the surface. The most severe type of open NTDs is craniorachischisis in which both the cranial and the spinal part of the neural tube did not close. When either the cranial or the spinal part of the neural tube are involved, the defects are named anencephaly and myeloschisis, respectively. Anencephaly, the lack of substantial parts of the brain, is generally believed to result from deterioration of exencephalic brain tissue that was present in the embryonic period. Thus exencephaly as observed

in laboratory animals is the equivalent of anencephaly in humans In the case of myeloschisis (also called spina bifida aperta) fluid may accumulate in the subarachnoid space ventral to the neural tissue, thereby pushing the neural tissue dorsally The resulting cystic appearance of this NTD is called myelomeningocele (meningomyelocele, spina bifida cystica) Since the open NTDs are caused by a failure of the neural tube to close, the period human embryos are susceptible to these defects ranges from the third till the end of the fourth week post conception In the case of closed NTDs the neural tube did form completely It is possible that these malformations arise from a faulty development of the mesenchymal tissue in the area of the neural tube These tissues are necessary for the formation of the meninges (arachnoid and dura), the vertebral arches and skull bones Failure of the formation of these tissues may result in a protrusion of meninges, which is covered by skin (meningocele, spina bifida cystica) When this defect occurs in the cranial region, the protruding mass may contain brain tissue, in which case it is named an encephalomeningocele In the least severe cases of closed NTDs the skeletal parts (vertebral arches or skull bones) did not completely close, however there is no protrusion of the meninges These defects are called spina bifida occulta and cranium bifidum occultum Since formation of the vertebral arches and skull bones starts at the fifth week after conception and continues until the early fetal period, this is the time window in which closed NTDs may be induced Albeit, ossification still has to occur later on and continues until after birth Spina bifida occulta in the sense of incomplete ossification of the vertebral arches is a normal appearance at birth and its persistence in the sacrum in 20 to 25% of the adults is considered a normal variation without clinical significance This thesis is concerned with the aetiology of open NTDs Therefore when the term NTD is used it refers to the open types of these defects

As stated above NTDs arise from a failure of the neurulation process From the previous section it is clear that many processes are involved in neurulation Since a disturbance of each of these processes may result in a NTD, it should be clear that the causes for NTDs are manyfold This is demonstrated by experiments in which rodent embryos have been subjected to substances interfering with processes involved in neurulation Decrease of the cellular ability to change its shape by inhibitors of microtubule function like colchicine and nocadazole, resulted in neural tube defects when mouse embryos were exposed *in vitro* (O'Shea, 1981, Smedley and Stanisstreet, 1986) Cytochalasins, agents which disrupt microfilament functioning and therefore decrease the cellular ability to change shape, also produced NTDs in mammalian embryos (Shepard and Greenaway, 1977, Wiley, 1980) NTDs can also be induced experimentally by inhibiting neural crest cell movement, as was demonstrated by Morriss and New (1979) when they exposed rat embryos to increased oxygen concentrations Disturbance of normal development of the paraxial mesenchyme may also lead to NTDs, *e g* by exposure to trypan blue (Morriss-Kay and Crutch, 1982,

Morriss-Kay and Tuckett, 1989; Peters and Dormans, 1981).

These examples of experimentally induced NTDs clearly demonstrate the involvement of several processes in neurulation. Still, the aetiology of 'naturally' occurring NTDs is poorly understood (Campbell *et al.*, 1986). Experiments as mentioned above reveal the processes we have to take into account. However, the factors that are responsible for the disturbances of processes essential to neurulation, are mostly unknown. They may be both genetic and environmental (Carter, 1969; Holmes *et al.*, 1976). Environmental and genetic factors may also act together as environmental factors may either increase or decrease the frequency of NTDs in the progeny given a certain genetic susceptibility. For example woman with an increased genetic susceptibility for NTDs due to a metabolic disorder related to folate metabolism might be more prone to give birth to an NTD affected child when their folate status is low than woman without the metabolic disorder. The other way around, their risk for a NTD affected pregnancy might be reduced by folate supplementation. The concept of folic acid preventable NTDs, and the experimental evidence that has led to it, will be discussed in the next section.

Prevention of neural tube defects by peri-conceptional folic acid supplementation

A possible link between folic acid and NTDs was first reported by Hibbard and Smithells (1965). Subsequently both observational and intervention studies have been undertaken, which have been reviewed elsewhere (e.g. Steegers-Theunissen *et al.*, 1993; Wald, 1994; Scott *et al.*, 1994). Although in some studies dietary intake was simply divided in categories like 'good' or 'poor' and in many studies peri-conceptional supplementation had occurred in the form of a multivitamin preparation, these studies suggested a key role for folic acid. Strongest evidence came from the MRC trial (MRC Vitamin Study Research Group, 1991). This study had a factorial design in which the recurrence risk for NTDs was investigated in four groups. The first receiving folic acid alone (4 mg daily), the second receiving a multivitamin preparation without folic acid, the third receiving both and the fourth receiving a placebo containing neither vitamins nor folic acid. It was found that peri-conceptional folic acid supplementation resulted in a 72% reduction of the recurrence risk for NTDs. The other vitamins did not show a significant reduction of the recurrence risk for NTDs. Since in other studies in which folic acid containing multivitamin preparations were used, the preventive effect was on average similar to the one in the MRC trial, the observed preventive effect in these studies may be ascribed to folic acid as well (Wald, 1994; Czeizel and Dudás, 1992).

Folic acid deficiency and neural tube defects

As peri-conceptional folic acid supplementation has a preventive effect on the risk for NTDs, the most simple explanation for this effect would be that folic acid supplementation relieves an existing folic acid deficiency. Of three studies investigating women with a folic acid deficiency so severe that they developed megaloblastic anaemia, two studies did not find that anaemic women were at higher risk for a NTD-affected pregnancy than healthy controls (Fraser and Watt, 1964; Giles, 1966; Pritchard *et al.*, 1970). Aminopterin, an anti-folate agent, has repeatedly been mentioned as a NTD-inducing agent in man. However as pointed out by Seller (1994) a wide variety of congenital malformations was observed and the case reported by Thiersh (1952) was the only one in which with certainty a NTD occurred. Nevertheless this case is also the only one in which exposure to aminopterin had occurred during the neurulation period.

Human studies investigating the folate status of mothers of NTD-affected children as compared to mothers of normal children gave inconsistent results. Whereas in some studies NTD-mothers did have a lower folate status in other studies no significant difference in folate status was found between cases and controls (Scott *et al.*, 1990). The time of sampling (e.g. during the index pregnancy or thereafter and early or late in the index pregnancy) and the use of the more probing method of measurement of red cell folate instead of measurement of serum folate, which reflects much more recent folate intake, are probable causes for the inconsistency of the results of the human studies on folate status. In a recent study by Kirke *et al.* (1993) with a large enough number of NTD cases (81) and controls (247) to detect subtle differences in folate status it was shown that even though the folate status of the cases was in the normal range, the frequency of NTDs was inversely related to the serum folate and red blood cell folate concentrations.

In laboratory animals the administration of anti-folates early in gestation rather led to increased resorption rates than to an increase of congenital malformations, and if the animals were exposed later in gestation congenital malformations varied depending on the time of exposure (Johnson and Chepenik, 1981). In mice a nutritional folate deficiency alone was insufficient to induce defective neurulation reproducibly (Heid *et al.*, 1992). Extreme folate deficiency in golden hamsters resulted in early embryonic loss (Mooij *et al.*, 1992).

In a study using the postimplantation rat embryo culture gestational day 9.5 rat embryos were cultured in serum from rats with a dietary folate deficiency (Miller *et al.*, 1989). Embryos were growth retarded and had many malformations, particularly of axial rotation (which is essential to rodent embryos to adopt the characteristic fetal conformation). Although development of prosencephalon and mesencephalon of rat embryos cultured in the serum from folate deficient

rats was inferior to the development of these brain vesicles in control embryos no NTDs were observed. Addition of N^5-methyltetrahydrofolate (5-methylTHF) to the serum reduced the number of malformed embryos, however they were still growth retarded, as expressed by their protein content. This indicates the dietary induced folate deficiency caused secondary effects which had a teratogenic action on their own that could not be overcome by increasing the folate supply *in vitro*.

Although some studies, especially the human studies, have been unsatisfactory, it can be concluded NTDs are not or seldom evoked by an overt folate deficiency, either due to administration of folate antagonists or due to nutritional deficiencies, but rather are caused by a folate-related phenomenon that is sensitive to a low folate status (although in the normal range) and can be counteracted upon by folic acid supplementation. Possibly an overt folate deficiency disturbs embryonic development to such an extent, that the conceptus is lost long before it is born.

Folate related metabolism

To understand which phenomena could be sensitive to a low folate status and might respond to folic acid supplementation, it is neccessary to have a closer look at the metabolic pathways that make use of or are related to folate.

Folate uptake and distribution

Folate (Fig. 7.3) is an essential nutrient for mammals. Dietary intake is primarily in the form of protein bound 5-methylpteroylpolyglutamates. Sufficient protease and conjugase activity are needed to convert them to the monoglutamate form, which is absorbed by the epithelial cells of the intestinal villi. Folic acid as present in food supplements is a monoglutamate form in which the pteroyl moiety is fully oxidized. Folic acid is absorbed by the intestinal cell as such and fully reduced to tetrahydrofolate (THF). In the intestinal cell the reduced folates are either released directly into the portal circulation, converted to 5-methylTHF or polyglutamated and released into the portal circulation. Since the intestinal reduction and methylation processes of folic acid are considered to be saturable, folic acid may be released unmodified into the circulation and first be metabolised in the liver when this compound is given as a food supplement at high doses (Lucock *et al.*, 1994). The monoglutamate form of 5-methylTHF is the principal circulating form of folate and therefore also the principal form by which the tissues are supplied with folate (Shane and Stokstad, 1983). The liver contains the main body store of folates, primarily in the polyglutamated form. Sarcosine synthase is the main folate binding enzyme. In other tissues folates can be polyglutamated as well, which has a function in the cellular retainment of

folate. For most of the enzymes of one-carbon metabolism that use folate derivatives, the polyglutamated folates are more effective substrates than the monoglutamated folates, and polyglutamation offers a potential regulatory mechanism in one-carbon metabolism (Shane, 1989).

The function of folate in one-carbon metabolism

The one-carbon substituted folates (Fig. 7.3) are used for various metabolic steps in which a carbon atom is transferred. The carbon atom is situated at the N^5- and/or N^{10}-site of the pteroyl moiety. It can be present in different oxidation states varying from 5-methylTHF to N^5-formyltetrahydrofolate (5-formylTHF) and N^{10}-formyltetrahydrofolate (10-formylTHF). The metabolic pathways of one-carbon metabolism are depicted comprehensively in Fig. 7.4. More concise illustrations of the relation of folate metabolism and the metabolism of homocysteine and methionine are Fig. 9.1 and Fig. 10.1.

The principal source of the carbon atoms is serine, which is converted to glycine by serinehydroxymethyltransferase. In this reaction tetrahydrofolate is converted to N^5,N^{10}-methylenetetrahydrofolate. Alternative sources are formiminoglutamic acid, a breakdown product in histidine catabolism and the mitochondrial glycine cleavage pathway and choline catabolism. The formation of 10-formylTHF from formate, ATP and THF is another possible way by which a carbon atom is attached to folate, however this pathway constitutes only a minor source of one-carbon units in mammalian tissues. Folate dependent one-carbon metabolism is used for purine and thymidine synthesis, histidine catabolism, the interconversion of glycine and serine, choline catabolism and the synthesis of methionine. This shows how much folate-dependent metabolism is intertwined with major metabolic pathways of the cell.

Methionine synthase and the methylation cycle

Folate enters the cell mostly in the form of 5-methylTHF. Methionine synthase is the only known enzyme capable of demethylating 5-methylTHF and is therefore a key enzyme to the cell's folate supply. The importance of the methionine synthase reaction for cellular folate levels becomes even more clear given the notion that 5-methylTHF is a poor substrate for folylpolyglutamate synthase and polyglutamation is necessary for cellular retainment of folate. Methionine synthase is also the enzyme by which folate metabolism is linked with the so called methylation cycle. The methyl group is transferred to the sulphur atom of homocysteine. The product of this reaction, methionine, is activated at the expense of ATP to the sulphonium ion S-adenosylmethionine (SAM). SAM is the universal substrate for methyltransferase reactions. There are 115 known different methyltransferase reactions that transfer the methyl group of SAM to a wide

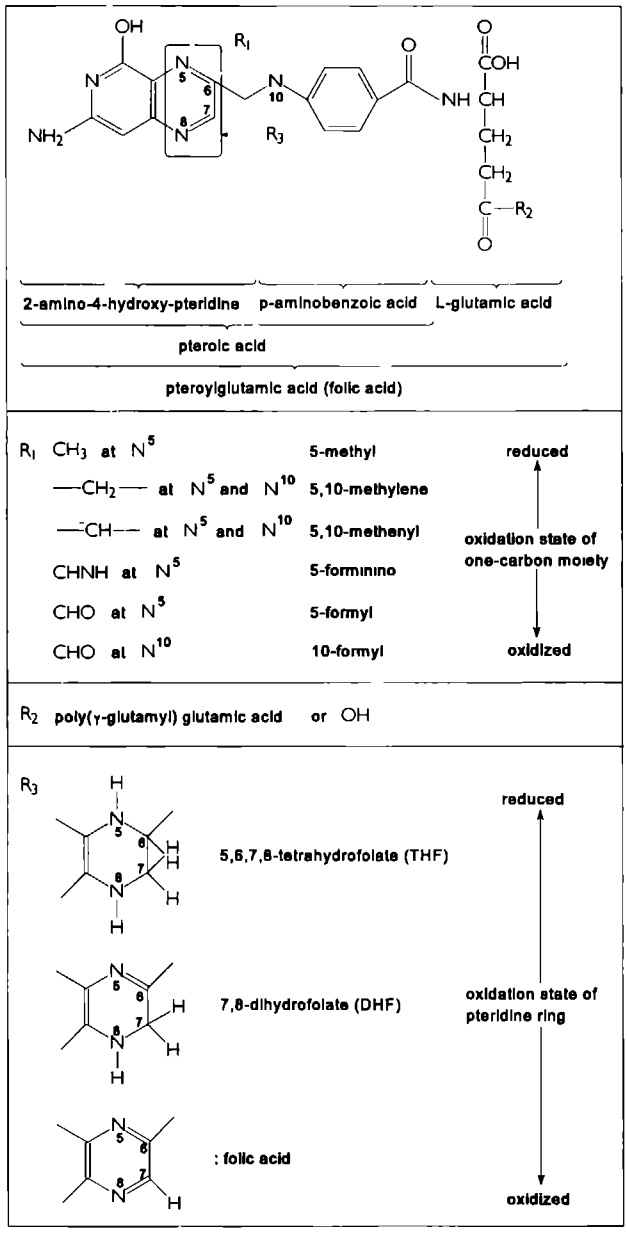

Fig. 7.3 Structure of folic acid and its derivatives Modified from Rosenblatt *et al.* (1989).

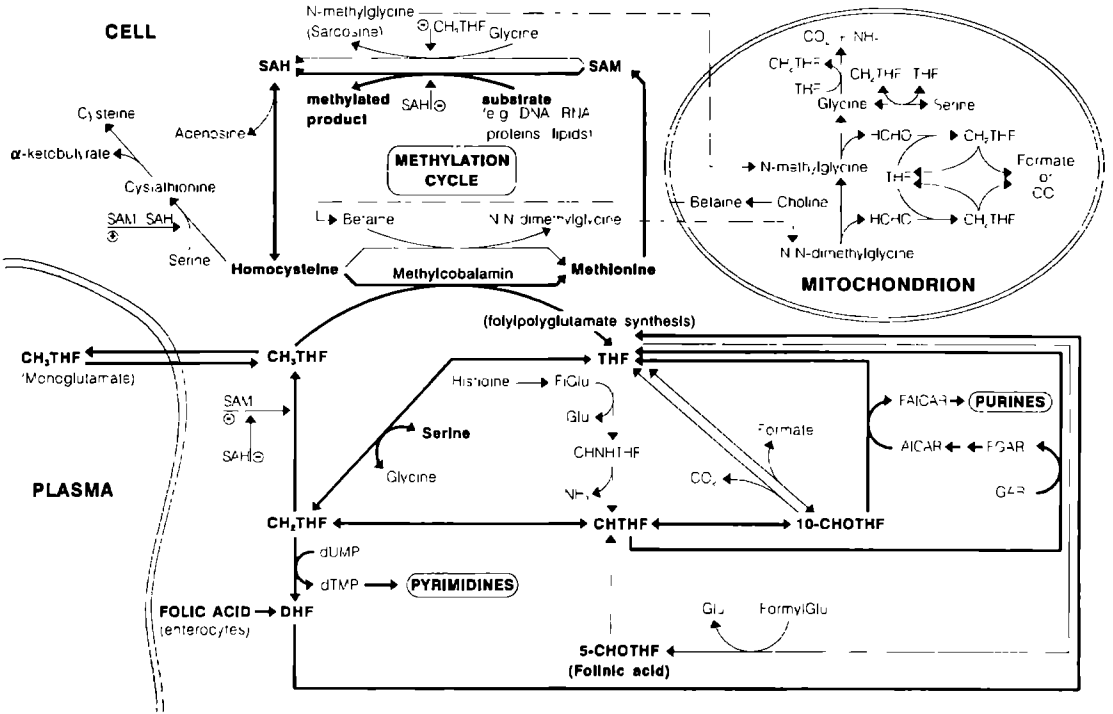

Fig. 7.4 Folate metabolism and its function in one-carbon transfer, modified from Scott *et al.* (1994) and Shane (1989) Co-substrates like electron acceptors and donors and ATP have been omitted. Legend on next page. Reprinted from VanAerts (1995). ©1995 Elsevier Science Ltd..

Legend to Fig 7.4 Perpendicular arrows show allosteric effectors that increase (⊕) or decrease (⊖) enzyme activity

DHF	= dihydrofolate	(F)AICAR	= 5-(form)amino-imidazole carboxamide ribonucleotide
CHTHF	= N^5,N^{10}-methenyl-tetrahydrofolate	(F)GAR	= (formyl)glycinamide ribonucleotide
CH_2THF	= N^5,N^{10}-methylene-tetrahydrofolate	(F_I)Glu	= (formimino)glutamate
CH_3THF	= N^5-methyltetrahydrofolate	FormylGlu	= formylglutamate
CHNHTHF	= N^5-formiminotetrahydrofolate	SAM	= S-adenosylmethionine
5-CHOTHF	= N^5-formyltetrahydrofolate	SAH	= S-adenosylhomocysteine
10-CHOTHF	= N^{10}-formyltetrahydrofolate	THF	= tetrahydrofolate
dTMP	= thymidine monophosphate		
dUMP	= deoxyuridine monophosphate		

range of substrates, including proteins, RNA, DNA and lipids (Scott *et al.*, 1994). Besides the methylated products, the methyltransferase reactions produce S-adenosylhomocysteine (SAH), which is an inhibitor of methyltransferases. SAH should therefore be quickly degraded, which is accomplished by S-adenosyl-homocysteine hydrolase. This hydrolysis can only occur when its products, homocysteine and adenosine, are sufficiently low in concentration. Adenosine is metabolised by adenosine deaminase. Homocysteine can either be remethylated to methionine or condensed with serine to yield cystathionine. The fate of homocysteine depends on the presence of the enzymes capable of metabolising homocysteine and on the need for SAM (see below). When homocysteine is remethylated the methylation cycle is complete. Homocysteine can be re-methylated by methionine synthase or by betaine-homocysteine methyl-transferase. The latter enzyme, however, is in most mammals only present in the liver and kidney.

Regulation of the methylation cycle

Methionine synthase is not the only link between folate metabolism and the methylation cycle. Regulatory feedback mechanisms are responsible for a more subtle intertwining of both (Finkelstein, 1990). Methyltransferase reactions depend on a sufficiently high SAM/SAH ratio. When SAM levels are high cystathionine-ß-synthase activity will be increased, which will result in increased breakdown of homocysteine through the transsulfuration pathway, thus leaving less homocysteine left to be remethylated. Also will high SAM levels inhibit methylenetetrahydrofolate reductase (MTHFR), thereby leading to a decrease of 5-methylTHF. The simultaneous decrease of the levels of both substrates for methionine synthase will lead to a reduced formation of methionine. When SAM levels are high most of it will be catabolized by sarcosine synthase (glycine-*N*-methyltransferase) (Balaghi *et al.*, 1993). This enzyme is inhibited by 5-methylTHF. The lowering of 5-methylTHF by high SAM levels will reduce this

inhibition, by which the breakdown of SAM will be stimulated Reversely, when SAM levels are low, the degree of transsulfuration will be reduced, the inhibition of MTHFR will be abolished, the formation of 5-methylTHF will be increased and sarcosine synthase will be inhibited Increased SAH levels will decrease the inhibitory effect of SAM on MTHFR and stimulate the activity of cystathionine-ß-synthase, thereby warranting an increased metabolism of homocysteine

Adult and embryonic folate metabolism

One-carbon metabolism as described above is mainly based on studies in liver tissues of adult rats and pigs Although most pathways are universal in mammals, considerable differences exist between adult tissues and fetal tissues For example cystathionine-ß-synthase activity is not present in rat embryonic tissue during neurulation (chapter 10 of this thesis) Methionine adenosyl transferase exists as three isozymes The low affinity type, apparently used for methionine catabolism when methionine concentrations are high, is only present in adult liver (Horikawa *et al*, 1993) Also differences in folate enzymes exist between embryonic and adult tissues These differences tend to favor one-carbon flow towards purine and pyrimidine synthesis at the expense of methionine synthesis in embryos (Shane, 1989)

Folate related metabolic disorders and neural tube defects

Yates *et al* (1987) first proposed a disordered folate metabolism as a basis for genetic predisposition to neural tube defects Their proposition was based on their discovery NTD-mothers not only had lower red cell folate levels as compared to controls, but also a difference existed between subjects and controls in the relationship between red cell folate and dietary folate intake In other words the cases - or a substantial portion of the cases - made a poorer use of the dietary folate supply as compared to the controls The hypothesis that this could be due to decreased activity of intestinal γ-carboxypeptidase - an enzyme that breaks down the polyglutamated folates to their absorbable monoglutamated forms - was investigated by Bower *et al* (1993) Their results did not support this hypothesis, but rather indicated distribution or metabolism of folic acid or its metabolites was impaired Results of another study of limited scale (Lucock *et al*, 1994) also suggested that NTD-mothers had a biochemical lesion somewhere in the multi-enzyme pathway leading from folic acid to 5-methylTHF

Other studies suggest low vitamin B12 plasma levels or a disordered transport or metabolism of vitamin B12 are associated with an increased risk for NTDs (Schorah *et al*, 1980, Gardiki-Kouidou and Seller, 1988, Magnus *et al*, 1991, Economides *et al*, 1992, Kirke *et al*, 1993) A recent study by Kirke *et al* (1993) showed an inverse relationship between both folate and B12 status and

the risk for a NTD-pregnancy Moreover, they showed there was a significant correlation between plasma B12 and red cell folate in cases, but not in controls Vitamin B12 in the form of methylcobalamin is the prosthetic group of methionine synthase Since 5-methylTHF is the main circulating form of folate and methionine synthase is the only known enzyme capable of demethylating 5-methylTHF, this enzyme is the key enzyme to the cell's folate supply Reduced activity of this enzyme would therefore be expected to result in a lower cellular folate level Kirke *et al* (1993) therefore suggested cases possessed an abnormal methionine synthase that is responsive to plasma vitamin B12 over a range where the normal enzyme would be fully functional and active However they did not exclude the possibility of a metabolic defect up- or downstream of methionine synthase, expressing itself through different rates of substrate flux through this enzyme between cases and controls Also a study by Schorah *et al* (1993) pointed in the direction of a reduced activity of methionine synthase in placental cytotrophoblasts from NTD affected pregnancies

Thus there is accumulating evidence both mildly impaired folate metabolism leading to reduced availability of 5-methylTHF and mildly impaired vitamin B12 metabolism possibly in combination with a low folate or vitamin B12 status are involved in the aetiology of folic acid-preventable NTDs The common denominator of such defects is a reduced substrate flux through the enzyme methionine synthase A reduced substrate flux through methionine synthas will lead to increased homocysteine levels and reduced methionine formation The investigation of the possibility that increased homocysteine levels are responsible for the increased risk for NTDs is the main objective of this thesis The concept of homocysteine toxicity will be shortly reviewed in the next section

Homocysteine toxicity

In 1962 Carson and Neill reported homocystinuria as a pathological condition in humans Mudd *et al* (1964) first showed a lack of cystathionine-ß-synthase activity in liver tissue of such a patient Due to the lack or diminished activity of this enzyme metabolism of homocysteine is decreased In untreated patients plasma homocysteine concentrations may reach levels of 0 2 mM, whereas in normal subjects total homocysteine concentrations in plasma are approximately 10 μM (Ueland *et al* , 1992) The main clinical manifestations are ectopic lenses, mental retardation, osteoporosis, dolichostenomelia and thromboembolism The latter of these symptoms is the major cause of morbidity in these patients. This complication is related to changes in vascular walls and perhaps enhanced clotting Since not only cystathionine-ß-synthase deficient patients show these vascular changes, but also homocystinuric patients due to a MTHFR deficiency or a disorder in cobalamin metabolism, who have high plasma homocysteine levels as well, but no hypermethionaemia, it has been hypothesized that the

elevated plasma and tissue homocysteine concentrations are the cause for the vascular pathology as observed in these patients (Mudd *et al.*, 1989).

Besides classic homocystinuria, which is the homozygous form of the autosomal recessively inherited cystathionine-ß-synthase deficiency, a slight increase in blood homocysteine levels, often designated as mild hyperhomocysteinemia, is a risk factor for premature vascular disease as well (Boers *et al.*, 1985; Clarke *et al.*, 1991). An often used method to identify such individuals is the oral methionine loading test. In this test post-loading plasma homocysteine levels are measured. Plasma homocysteine concentrations more than two standard deviations above the mean of a control population is regarded as positive with regard to a disturbance in methionine metabolism. Heterozygosity for cystathionine-ß-synthase deficiency as a possible cause for the observed mild hyperhomocysteinaemia can only be concluded after measuring cystathionine-ß-synthase activity in a cell culture derived from the subject, e.g. a fibroblast culture. The increased blood homocysteine levels may also be caused by a low folate or vitamin B6 or B12 status or disordered folate or vitamin B12 metabolism.

Similarly Steegers-Theunissen *et al.* (1991) reported that NTD mothers frequently showed a derangement of methionine metabolism as diagnosed by the methionine loading test. Cystathionine-ß-synthase deficiency was not found in these women, nor were the vitamin levels of these women out of the normal range (Steegers-Theunissen *et al.*, 1994). This suggested these women had a disorder in folate or vitamin B12 metabolism which led to increased homocysteine levels. As has been discussed above folic acid supplementation can prevent NTDs. Folic acid therapy is also an established way to correct homocysteine levels in hyperhomocysteinaemic patients (Refsum and Ueland, 1990). It was therefore hypothesized that the increased maternal or embryonic homocysteine concentrations could be an aetiologic factor in the induction of folic acid-preventable NTDs and that the mechanism of folic acid-mediated prevention of NTDs could be based on a correction of a mild hyperhomocysteinaemic state. In the studies presented in the next three chapters this hypothesis is investigated.

Stereospecific *In Vitro* Embryotoxicity Of L-Homocysteine In Pre- And Post-Implantation Rodent Embryos[1]

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Toxicology in vitro 7:743-749

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Abstract Recently a derangement of homocysteine metabolism has been suggested as a possible risk factor for neural tube defects and recurrent spontaneous abortion. To investigate a possible role of homocysteine in the aetiology of neural tube defects we tested the *in vitro* embryotoxicity of L-homocysteine by culturing day 10 *post coitum* post-implantation ratembryos in whole embryo culture (WEC) for 24 hours and day 2 *post coitum* pre-implantation mouse embryos for 48 hours. With an area under curve (AUC) of 6.3 mM·hr L-homocysteine significantly reduced the percentage of mouse embryos that developed into blastocysts. In rat WEC an AUC for L-homocysteine of 3.6 mM·hr reduced the mitotic index of the neural epithelium of the rhombencephalon and the cell density of the mesenchyme adjacent to it, while at an AUC of 7.2 mM·hr L-homocysteine reduced the total morphological score (TMS) and the number of dysmorphogenic features per conceptus was increased. Dysmorphogenic features most often seen were transparent rhombencephalon, no or delayed forelimb bud formation, dysmorphogenesis of the somites and blister formation dorso-laterally of the place of forelimb bud formation. The embryotoxicity of L-homocysteine was stereospecific since D-homocysteine caused no embryotoxic effects. Also the oxidation product L-homocystine (AUC, 72 mM·hr) and the metabolite L-methionine (AUC, 144 mM·hr) were not embryotoxic. Both stereoisomers of homocysteinethiolactone were embryotoxic at an AUC of 72 mM·hr. The results are discussed in relation to the metabolism of homocysteine and methionine and their possible role in the neurulation process.

Introduction

Recently a derangement of homocysteine metabolism has been suggested as a possible risk factor for neural tube defects (NTDs) and recurrent spontaneous abortion (Steegers-Theunissen *et al.*, 1991; 1992). The underlying mechanism is

[1]See note on page 9

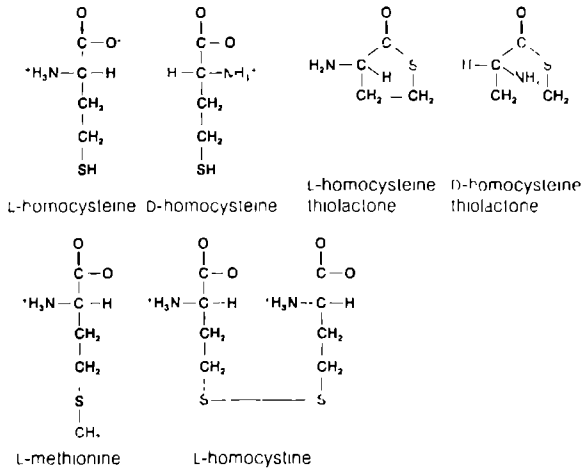

Fig. 8.1. Structural formulae of L-homocysteine and related compounds tested in rat whole embryo culture.

not clear, but the fact that patients show abnormally high blood levels of homocysteine after oral methionine loading indicates that homocysteine disposal is at risk. Hyperhomocysteinaemia is an established risk factor for premature vascular disease (Boers *et al.*, 1985; Clarke *et al.*, 1991). Pathological accumulation of homocysteine in tissues and blood of cystathionine-ß-synthase deficient patients is generally considered to initiate the vascular occlusive diseases from which these patients suffer (Mudd *et al.*, 1989). Besides inherited metabolic disorders like cystathionine-ß-synthase deficiency and disorders in conversion and handling of cobalamin species, vitamin B12 deficiency and folate deficiency may also lead to accumulation of L-homocysteine (Mudd *et al.*, 1989; Refsum and Ueland, 1990). Intriguing in this respect is that these deficiencies also have been associated with the occurrence and recurrence of NTDs (Smithells *et al.*, 1980, 1983; Shorah *et al.*, 1980; Laurence *et al.*, 1981; Gardiki-Kouidou and Seller, 1988; MRC Vitamin Study Research Group, 1991). Therefore we were prompted to test the embryotoxicity of L-homocysteine and related compounds (Fig. 8.1) in order to investigate the possible role of L-homocysteine in the aetiology of NTDs.

Whole embryo culture (WEC) has been used to investigate embryotoxic effects of deficiencies as well as excess of amino acids (Denno and Sadler, 1990; Hamers *et al.*, 1989; Cockroft, 1988; Coelho *et al.*, 1989). Therefore we used WEC to test the embryotoxicity of L-homocysteine during neurulation. We also used pre-implantation mouse embryo culture to test the embryotoxicity of L-homocysteine during early embryonic development.

Materials and methods

Chemicals

Homocysteinethiolactones and amino acids were purchased from Sigma (St. Louis, MO, USA). Hormones were purchased from Organon (Oss, The Netherlands).

WEC medium preparation

Blood was taken from adult volunteers (male and female) between 20 and 50 yr of age. The blood was defibrinized during donation by shaking with glass pearls in a glass bottle. The defibrinized blood was centrifuged immediately, the serum was heat-inactivated (30 min, 56 °C) and D-glucose was added (720 mg/l). After testing each individual serum for compatibility with WEC, the serum of two to five individuals was pooled and stored at -20 °C. The WEC medium consisted of 75% serum and 25% Tyrodes salt solution. All substances added to the medium were dissolved in Tyrodes salt solution Homocysteine was freshly prepared from homocysteinethiolactone by alkaline hydrolysis (5 min, 37 °C, 5 N NaOH).

Thiol concentrations

Homocysteine concentrations were assayed by measurement of the thiol concentrations with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

Whole embryo culture

Method Random bred Cpb.WU (Wistar) rats were housed two or three to a cage and were allowed to eat (MRH-TM pellets, Hope Farms B.V., Woerden, The Netherlands) and drink (tap water) *ad lib.*. Lights were on from 12.00 until 00.00 hr. On day 0 females (11-26 wk old, 200-300 g), ready to mate, were housed together with males, one to one, from 9.00-12.00 hr. Embryos were explanted between 13.30 and 15.30 hr on day 10. Only embryos with 4-8 somites and without any abnormalities were used for WEC. The embryos were cultured for 24 hr in a 25 ml glass bottle containing 2 ml medium at 37.5 °C and rotated at 30 rpm. The culture bottles were gassed with a mixture of 5% O_2, 90% N_2 and 5% CO_2. After 2 and 18 hr of culture the bottles were gassed with a mixture of 20% O_2, 75% N_2 and 5% CO_2.

Morphological and histological endpoints. After culturing, the morphology of the embryos was evaluated as described by Brown and Fabro (1981). All dysmorphogenic features were recorded. Four embryos from each group were examined histologically. The embryos were fixed in Bouin's fluid, dehydrated with ethanol and chloroform and embedded in paraffin. The head and rump were treated separately as described by Langenfeld *et al.* (1988). 4 μm slices were taken at 20 μm intervals and stained with haematoxylin and eosine. Besides general histological evaluation the mitotic indices of the neural epithelium of the rhombencephalon and the cell densities in 2500 μm^2 areas of the mesenchyme adjacent to both sides of the rhombencephalon ventrally of the otic primordia were measured in three consecutive slices.

Pre-implantation mouse embryo culture

Random bred female Cpb:SE (Swiss) mice were housed in cages (5-20 animals in each

cage) and could eat (MRH-TM pellets, Hope Farms B.V.) and drink (tap water) *ad lib.*. Males were housed separately. Lights were on from 06.00 until 18.00 hr. To provoke a superovulation females were injected *i.p.* with Humegon followed 48 hr later with Pregnyl (10 U in 0.1 ml 0.9% NaCl of each). Females were housed, one to one, with males and were checked for the presence of a vaginal plug the next morning. Two days *post coitum* embryos were flushed from the oviducts, checked for normal morphology and cultured for two days in human tubal fluid-like (HTF) medium (Quinn *et al.*, 1985) containing 0.5% bovine serum albumine in an atmosphere containing 5% CO_2 with 100% humidity at 37 °C. After culturing the percentage of embryos that developed into blastocysts was determined.

Statistical analysis

Differences between groups were tested with Student's *t*-test with Bonferroni correction. With a nominal α of 0.05, differences were regarded as statistically significant at $P < 0.05/k$ where k is the number of comparisons made. Least square means of TMS and number of dysmorphogenic features per conceptus were estimated by analysis of covariance, taking the number of somite pairs at the beginning of culture as covariant. All calculations were performed with SAS statistical software v. 6.06 on a Digital VAX 6000-410 computer.

Results

Homocysteine oxidation

During culturing of the embryos, homocysteine oxidized spontaneously to its disulfide homocystine. The pseudo-first-order rate constants of homocysteine oxidation were 70×10^{6} s^{-1} in WEC and 3.2×10^{-6} s^{-1} in HTF medium. These rate constants correspond to half-life values of 2.8 hr in WEC and 60 hr in HTF medium. As a result of this oxidation homocysteine disappears from the medium. We therefore considered it more appropriate to use the AUC as a measure of exposure.

Post-implantation rat embryo culture (WEC)

Morphology. In WEC, L-homocysteine was embryotoxic at an AUC of 7.2 mM·hr as was demonstrated by a decreased TMS and an increase in the number of dysmorphogenic features per conceptus (Table 8.1). Dysmorphogenic features most often seen were enlarged and transparent rhombencephalon, no or delayed formation of forelimb buds, irregular and reduced number of somites and blisters - sometimes filled with blood - dorso-laterally near the place of forelimb bud formation (Fig. 8.2).

Histology. With L-homocysteine at an AUC of 7.2 mM·hr many histological abnormalities were observed, especially in the rump part. The neural tube was deformed and much smaller and extensive necrotic foci and many apoptotic

AUC (mM·hr)	Initial concn (mM)	TMS†	Dysmorphogenic features‡	Mitotic index§	Cell density¶
0	**0**	41 8 (0 7)	0 4 (0 4)	4 1 (0 5)	12 9 (0.1)
1.1	**0.27**	43.5 (0.7)	0 1 (0.4)	3.7 (0.4)	12 0 (0 7)
3.6	**0.9**	42 3 (0 7)	0 6 (0 4)	2 9 (0.2)*	10 3 (0.8)**
7.2	**1.8**	37 1 (0.7)**	5 0 (0.4)**	2.6 (0.2)*	9.0 (0 5)**
10.8	**2.7**	30.3 (0.7)**	7 0 (0.4)**	n.d.	n d.

Table 8.1. Embryotoxicity of L-homocysteine in post-implantation rat embryo culture. Values are least square means and, in parentheses SEM Those marked with asterisks differed significantly (Students *t*-test) from the corresponding control values: *P< 0 05 **P<0 005.

† Total morphological score (n=13)

‡ No of dysmorphogenic features per conceptus (n=13).

§ Mitotic indices of neural epithelium of rhombencephalon (n=4).

¶ Cell densities of mesenchyme adjacent to rhombencephalon (cells/2500 μm^2, n=4).

n d = not detectable

bodies were present throughout the embryos (Fig. 8.3). Although at an AUC of 3.6 mM·hr L-homocysteine no gross histological changes were apparent, both the mitotic indices of the neural epithelium of the rhombencephalon and the cell densities of the mesenchyme adjacent to it were reduced significantly (Table 8.1).

Specificity of L-homocysteine embryotoxicity. When the embryotoxicity of L-homocysteine was compared with that of related compounds it was shown that homocysteine embryotoxicity was stereospecific: whereas L-homocysteine was very toxic (AUC 10.8 mM·hr) D-homocysteine caused no embryotoxic effects at all (Table 8.2). Also the oxidation product L-homocystine (72 mM·hr) and L-methionine (AUC 144 mM·hr) were not embryotoxic. However the condensed forms of both stereoisomers of homocysteine - D- and L-homocysteine-thiolactone - were embryotoxic at an AUC of 72 mM·hr (Table 8.2). The thiolactones induced the same abnormalities as L-homocysteine. However at the concentrations used the formation of large blisters near the region of forelimb bud formation was more pronounced and more often seen after exposure to the thiolactones (Fig. 8.2).

Pre-implantation mouse embryo culture

L-Homocysteine was also toxic for pre-implantation mouse embryos. At an AUC of 6.3 mM·hr (corresponding with an initial concentration of 170 μM) the num-

Compound	AUC (mM·hr)	Initial concn (mM)	TMS†	Dysmorphogenic features‡
Control	-	-	44 5 (0 8)	0 4 (0 6)
L-homocysteine	10.8	2.7	35 6 (0 8)*	7 9 (0 6)*
D-homocysteine	10.8	2.7	43 3 (0 8)	0 4 (0 6)
L-homocysteinethiolactone	72	3	29 8 (0 8)*	12 3 (0 6)*
D-homocysteinethiolactone	72	3	32 6 (0 8)*	10 7 (0 6)*
L-homocystine	72	3	42 7 (0 7)	0 6 (0 5)
L-methionine	144	6	43 6 (0 8)	0 1 (0 6)

Table 8.2. Embryotoxicity of L-homocysteine and structural analogues in post-implantation rat embryo culture Values are least square means and, in parentheses, SEM Values marked with an asterisk differed significantly (Students*t*-test) from the corresponding control value *P<0 007
† Total morphological score (n=12)
‡ No of dysmorphogenic features per conceptus (n=12)

ber of embryos that developed into blastocysts was reduced approximately four-fold (Table 8.3).

Discussion

Our experiments clearly demonstrate that L-homocysteine is embryotoxic *in vitro*. Since its enantiomer D-homocysteine, its oxidized form L-homocystine and its metabolite L-methionine are not embryotoxic, it is evident that homocysteine embryotoxicity is not caused only by the thiol or amino acid moiety of L-homocysteine, but requires the intact molecule in a stereospecific manner.

AUC (mM·hr)	Initial concn (mM)	N	% blastocysts
0	0	64	67 (12)
0.63	0.017	38	76 (23)
1.9	0.051	36	66 (15)
6.3	0.17	37	18 (13)**
19	0.51	25	0**

Table 8.3. Embryotoxicity of L-homocysteine in pre-implantation mouse embryos Values are means and, in parentheses SD Values marked with asterisks differed significantly (Students *t*-test) from the corresponding control values **P<0 001

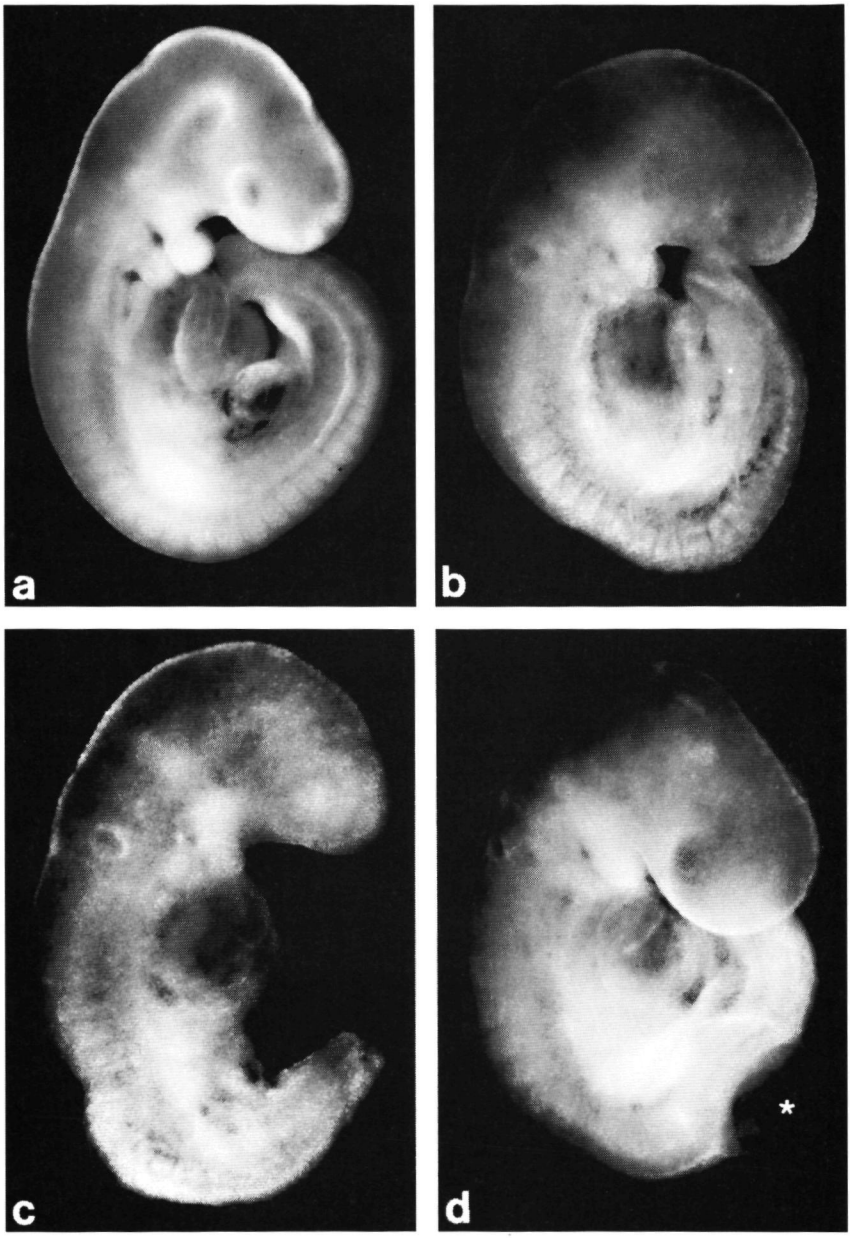

Fig. 8.2. Rat embryos cultured *in vitro* from day 10 of gestation for 24 hr (×23). (**a**) Control. (**b**) L-Homocysteine AUC = 7.2 mM·hr. (**c**) L-homocysteine AUC = 10.8 mM·hr. (**d**) D-Homocysteinethiolactone AUC = 72 mM·hr, showing large blister (*).

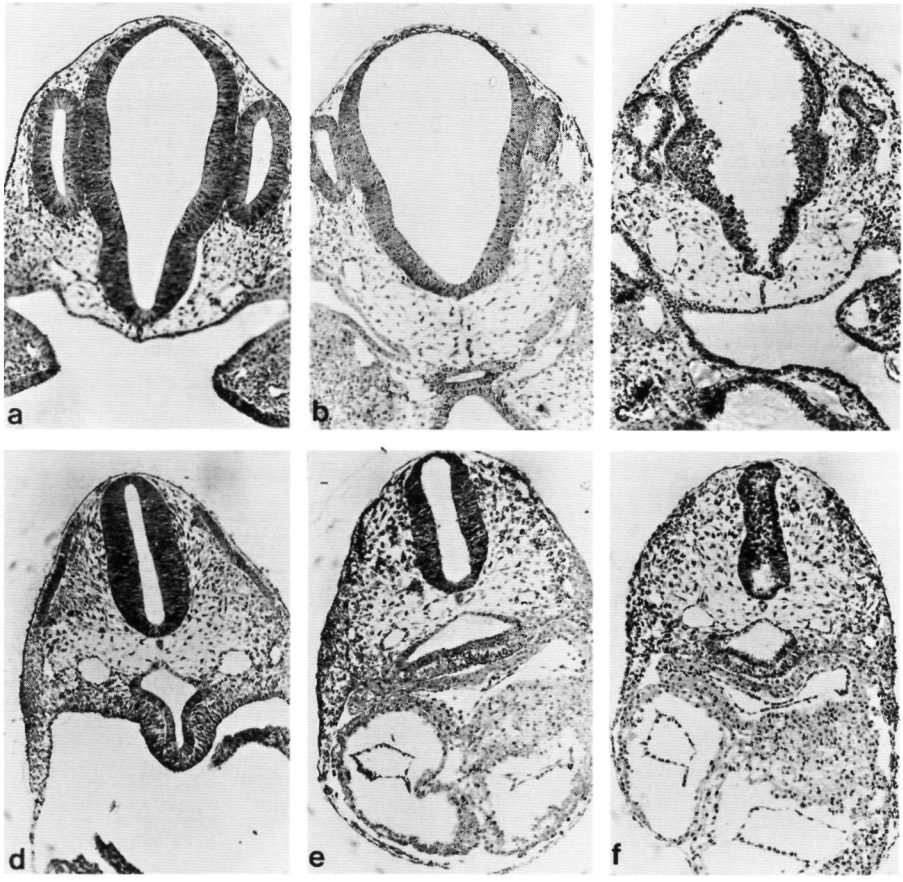

Fig. 8.3. HE-stained slides of rat embryos cultured *in vitro* for 24 hours (×68). (**a**), (**b**), (**c**): Transversal section through rhombencephalon and otic primordia. (**d**), (**e**), (**f**): Transversal section through rump part showing heart and neural tube. (**a**), (**d**): Control. (**b**), (**e**): L-Homocysteine AUC = 7.2 mM·hr. (**c**), (**f**): L-Homocysteine AUC = 10.8 mM·hr.

A surprising result was the observation that the stereospecificity of L-homocysteine embryotoxicity is not matched by a stereospecific embryotoxicity of homocysteinethiolactone. This result may be interpreted as a difference in transport mechanism. While L-homocysteine is dependent on a stereospecific carrier to enter the cell, the thiolactones may - because of their reduced polarity - simply enter the cell by diffusion.

The initial concentrations of L-homocysteine used in WEC are much higher than those found in the sera of controls and of women showing a positive result in the methionine loading test (Steegers-Theunissen *et al.*, 1991). There are several explanations of why these high concentrations of L-homocysteine were needed to cause embryotoxicity. First, L-homocysteine when added to the medium disappears rapidly because of its spontaneous oxidation to L-homocystine, which is not embryotoxic (Table 8.2). The apparent difference in sensitivity to L-homocysteine embryotoxicity between pre-implantation mouse embryos and post-implantation rat embryos can be explained by a difference in the rate of L-homocysteine oxidation. Secondly, it might be difficult to increase the intracellular homocysteine concentration by increasing the medium concentration. Indeed it has been hypothesized that cells possess an efficient export mechanism for L-homocysteine to ensure low intracellular L-homocysteine concentrations which may be essential to some vital cellular functions (Svardal *et al.*, 1986).

The lower mitotic indices of the rhombencephalon and cell densities of the mesenchyme adjacent to it indicate that at an AUC of 3.6 mM·hr L-homocysteine inhibits cell proliferation. No NTDs were observed in the embryos cultured in our experiments. This may be explained by the stage of development of the embryos. When 9.5-day-old rat embryos were cultured in cow serum, which is methionine deficient, they failed to close their neural tube. However the necessity for methionine supplementation exists only during the first 18 hr of culture (Coelho *et al.*, 1989). When Matsuda and Yasutomi (1992) added 0.8 μM 5-azacytidine to a culture of rat embryos (9.5 days of gestation) incomplete closure of the cephalic neural tube was observed. Maximum sensitivity occurred between 6 and 12 hours of culture. Thus it is plausible that the best period to induce NTDs in rat embryos is before day 10 of gestation. In our experiments the embryos were explanted at 10 days of gestation.

From our results it is not clear what mechanism is responsible for the observed toxic effects of L-homocysteine. Since D-homocysteine is not embryotoxic it is not very likely that embryotoxicity is induced simply by a high concentration of oxidizable free thiols in the medium. The hypothesis therefore that the mechanism is similar to that proposed for homocysteine toxicity to endothelial cells, where copper-catalysed oxidation of homocysteine leads to hydrogen peroxide formation (Starkebaum and Harlan, 1986) is rather improbable. When we view our results alongside those of Coelho and co-workers (Coelho and Klein, 1990: Coelho *et al.*, 1989) and Matsuda and Yasutomi (1992) we suggest the follow-

ing mechanism. Both methionine deficiency and excess of homocysteine may lead to a decrease of the ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) (Finkelstein, 1990). A reduced SAM/SAH ratio will inhibit numerous transmethylation reactions in which SAM is the methylgroup donor. One of the transmethylation reactions is involved in the methylation of amino acid residues in actin and myosin fibrils. A reduced methylation index of these amino acid residues in neural tube proteins was observed by Coelho and Klein (1990) when embryos were cultured in methionine-deficient cow serum. Another transmethylation product is 5-methylcytosine. This methylated nucleotide occurs in very specific places on the DNA and methylation of these sites may play a role in the regulation of the expression of tissue specific genes (Cedar, 1988). Matsuda and Yasutomi (1992) showed that hypomethylation of DNA occurred in embryos that failed to close their neural tube after exposure to 5-azacytidine.

The *in vitro* embryotoxicity of L-homocysteine that we have demonstrated in the present work is in accordance with previous reports that a deranged homocysteine metabolism could possibly be an aetiological factor in NTDs or recurrent spontaneous abortion (Steegers-Theunissen *et al.*, 1991; 1992). However, considering the concentrations of L-homocysteine used in our experiments and the levels of maternal plasma L-homocysteine it is not very probable that it is the maternal homocysteine that causes the problems for the embryo. Further investigations will be needed to elucidate the role of homocysteine and methionine metabolism in the aetiology of NTDs.

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Prevention Of Neural Tube Defects By And Toxicity Of L-Homocysteine In Cultured Postimplantation Rat Embryos

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Teratology 50:348-360

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Abstract Mild hyperhomocysteinemia is frequently observed in mothers who gave birth to a child with a neural tube defect (NTD). In a previous study we showed L-homocysteine was embryotoxic to gestational day 10 rat embryos in culture, however no NTDs were observed. We therefore investigated the effect of L-homocysteine on the development of neural plate stage (gestational day 9.5) rat embryos. Other objectives of this study were investigation into whether the embryotoxicity of L-homocysteine could be attenuated by compounds related to its metabolism and clarification of the mechanism of L-homocysteine embryotoxicity. In gestational day 9.5 rat embryos L-homocysteine was not toxic at 1 and 2 mM concentrations. Rather at these concentrations it promoted development of the rat embryos in serum that without supplementation caused neural tube defects in the embryos. L-Methionine had the same preventive effect at even lower concentrations, but folinic acid (1 mM) did not improve embryonic development. N^5-Methyltetrahydrofolate (5-methylTHF) (100 μM), L-serine (6 mM) and L-methionine (6 and 12 mM) attenuated the embryotoxicity of L-homocysteine (6 mM) in gestational day 10 rat embryos. Vitamin B_{12} (10 μM) completely abolished the embryotoxicity of L-homocysteine, which was shown to be mediated by catalysis of the spontaneous oxidation of L-homocysteine to the less toxic L-homocystine. In gestational day 11 rat embryos both L- and D-homocysteine were readily taken up when added to the culture (3 mM) and increased embryonic S-adenosylhomocysteine (SAH) levels 14 and 3-fold, respectively. This difference was shown to be caused by the stereospecific preference of SAH hydrolase. We propose the basis for L-homocysteine embryotoxicity is an inhibition of transmethylation reactions by increased embryonic SAH levels.

Introduction

Several findings point to the crucial role of a disturbed homocysteine/methionine metabolism in the aetiology of NTDs. First, peri-conceptional folate supplementation reduces both the NTD first occurrence and recurrence risk significantly (Czeizel and Dudás, 1992; MRC Vitamin Research Group, 1991). Folate deficiency or a disturbed folate metabolism, leading to a reduced availability of 5-

methyltetrahydrofolate (5-methylTHF), will result in decreased homocysteine remethylation, and therefore increases homocysteine levels and decreases methionine levels (Mudd *et al*, 1989) (Fig. 9.1). Second, there is growing evidence that, next to folate, vitamin B12 may play a role in the aetiology of NTDs as well (Schorah *et al.*, 1980; Gardiki-Kouidou and Seller, 1988; Magnus *et al.*, 1991; Economides *et al*, 1992; Kirke *et al.*, 1993). Methionine synthase, the enzyme remethylating L-homocysteine to L-methionine is a vitamin B12-dependent enzyme. The studies by Schorah *et al.* (1993) pointed in the direction of a reduced activity of methionine synthase in placental cytotrophoblasts from NTD-affected pregnancies. Third, in mothers who gave birth to a child with NTD, homocysteine blood levels were on average higher as compared to controls, both before and after an oral methionine loading test, indicating a defective homocysteine/methionine metabolism (Steegers-Theunissen, 1993). In women in whom the oral methionine loading test was abnormal no deficiency of the homocysteine metabolizing enzyme cystathionine-ß-synthase could be found. (Steegers-Theunissen *et al.*, 1994) It is therefore more likely that the remethylation of homocysteine to methionine is reduced. L-methionine deficiency has been shown to induce NTD in cultured rat embryos (Coelho *et al*, 1989; Coelho and Klein, 1990) and methionine supplementation has been shown to promote remediation of axial defects in the mouse (Essien 1992; Essien and

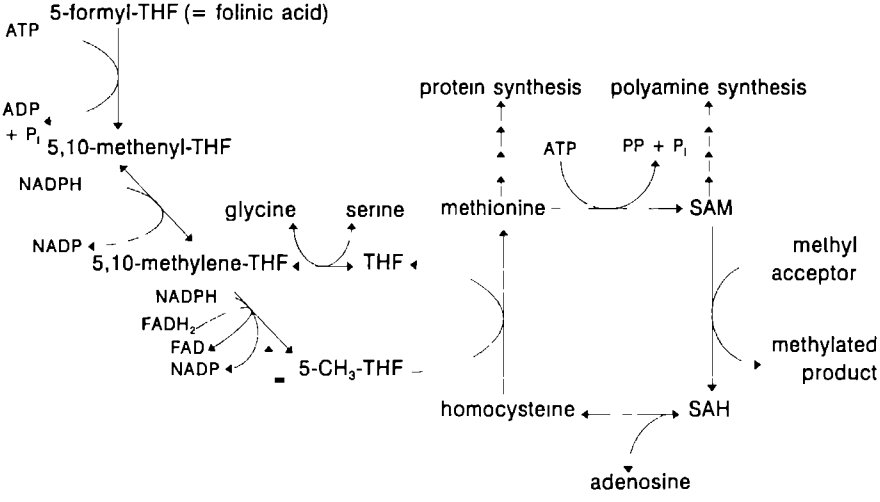

Fig. 9.1 Simplified metabolic scheme illustrating homocysteine/methionine metabolism and its relation to folate metabolism and transmethylation as far as it is discussed in this paper SAM, S-adenosylmethionine, SAH, S-adenosylhomocysteine, THF, tetrahydrofolate

Wannberg, 1993a,b) and the attenuation of valproate toxicity in the rat (Nosel and Klein 1993) Finally, in a previous study we have shown, that L-homocysteine is embryotoxic to gestational day 10 rat embryos *in vitro* (VanAerts *et al*, 1993a)

In this paper we present several experiments concerning the effects of L-homocysteine and compounds related to its metabolism on embryonic development and homocysteine metabolism, using the post-implantation rat embryo culture Since no NTDs were observed when gestational day 10 rat embryos were cultured in the presence of toxic levels of L-homocysteine (VanAerts *et al*, 1993a) we cultured neural plate stage (gestational day 9 5) rat embryos in the presence of L-homocysteine, L-homocystine, L-methionine and folinic acid To investigate whether L-homocysteine embryotoxicity could be attenuated by stimulation of its metabolism we added 5-methylTHF, L-serine, vitamin B12 and L-methionine to the culture of gestational day 10 rat embryos We added catalase and Cu(II)SO4 to test whether a H2O2 mediated mechanism was involved in L-homocysteine embryotoxicity To examine if a disturbance of homocysteine/methionine metabolism by elevated L-homocysteine concentrations caused the embryotoxicity, we investigated the effect of 3 mM L- and D-homocysteine and their thiolactones on the levels of S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), methionine and homocysteine in gestational day 11 rat embryos Finally the enzyme kinetics of SAH hydrolase were studied

Materials and methods

Whole embryo culture

To investigate the effects of elevated L-homocysteine, L-homocystine, L-methionine and folinic acid concentrations on morphological development of conceptuses at an earlier stage of development, conceptuses were explanted at 9 5 days of gestation (early to late neural plate stage, [Fujinaga and Baden, 1991]) Reichert's membrane was removed by the traditional method, *i e* leaving the ectoplacental cone intact Conceptuses were cultured, two in a bottle, for 48 hr in 2 ml medium Medium consisted of 71% human serum (preparation according to VanAerts *et al*, [1993a]), 10% rat serum (preparation according to Piersma *et al* [1991]) and 19% Tyrode's buffer In this experiment we added rat serum, since human serum, compatible with embryonic development of gestational day 10 rat conceptuses, did not support embryonic development of gestational day 9 5 conceptuses Penicillin G, sodium salt, (6 μg/ml), streptomycinesulfate (0 66 mg/ml), D-glucose (final concentration 3 mg/ml) and test substances were dissolved in Tyrode's buffer before addition Test substances were added after preparation of the embryos The culture bottles were gassed initially and after 4 hr of culture with 5% O2, 90% N2 and 5% CO2 After 20 and 28 hr the bottles were regassed, with the oxygen concentration increased to 20% at the expense of nitrogen After 44 hr, the oxygen concentration was increased to 40% Housing of the animals and the conditions of culture were described previously (VanAerts *et al*, 1993a) Total morphological score

(TMS) was assessed by the method of Brown and Fabro (1981) and all dysmorphogenic features were recorded

To test the modulation of L-homocysteine embryotoxicity by L-methionine, 5-methyl-THF, L-serine, vitamin B12, Cu(II)SO4 and catalase, gestational day 10 rat conceptuses (four to eight pairs of somites) from random-bred Cpb WU Wistar rats were cultured for 24 hr, one in each bottle The medium consisted of 75% human serum and 25% sterile filtered Tyrode's buffer, in which all substances added were dissolved Human serum was preferred above rat serum, to reduce the number of animals used Each individual serum was tested in the gestational day 10 embryo culture for compatibility with embryonic development and those sera supporting embryonic development were pooled (four to six individual sera per pool) Within each experiment only one pool was used After addition of the test substances the media were pre-incubated for 1 5 to 3 5 hr before embryos were added The time of pre-incubation was equally spread among the experimental groups The conceptuses were evaluated in the same way as gestational day 9 5 conceptuses

Gestational day 11 or gestational day 11 5 conceptuses were used for the experiments in which embryonal homocysteine, methionine, SAM and SAH concentrations were investigated Older embryos were used for this experiment because physiological levels of homocysteine and SAH are very low and the small biomass of gestational day 9 5 or gestational day 10 embryos would have required the use of very large numbers of embryos After preparation of the gestational day 11 conceptuses the test substances were added and the conceptuses were cultured, two or three in a bottle, for 2 hr in 2 ml medium, which consisted of 75% human serum and 25% Tyrode's buffer and were gassed with 40% O2, 55% N2 and 5% CO2 We used a shorter incubation period to prevent complete oxidation of homocysteine to its disulfide After culture conceptuses were washed three times in ice-cold Hank's buffered salt solution (HBSS) To measure the levels of methionine, SAM, SAH and total homocysteine in embryos proper the embryonic membranes (yolk sac and amnion) were removed and the embryos proper were washed once again in HBSS After washing the material was frozen in liquid nitrogen and stored at -80 °C To compare *in vivo* and *in vitro* levels of metabolites, gestational day 11 5 rat conceptuses were either immediately frozen after explantation and removal of decidual tissue, Reichert's membrane and - in the case of measurement of metabolites in embryos proper - yolk sac and amnion, or were cultured for 2 hr as described for gestational day 11 embryos

Homocysteine oxidation

The rate of homocysteine oxidation was assayed under the conditions of gestational day 10 embryo culture (oxygen concentration 5%) and was assessed by measuring the free thiol concentration according to Ellman (1959) The data obtained in this way were fitted to a curve with the general expression

$$[\text{homocysteine}]_T = [\text{homocysteine}]_0 \cdot e^{(-k' T)},$$

in which k' is the pseudo-first order rate constant of oxidation and T the time elapsed

S-adenosylhomocysteine hydrolase assay

SAH hydrolase was assayed in homogenate of gestational day 11 5 embryos proper that had been frozen in liquid nitrogen immediately after preparation and were stored at -80

°C Tissues were sonicated in an ice-cold 22 5 mM potassium phosphate buffer (pH 7 0) containing 1 mM Na2EDTA 2H2O, 20 μM erythro-9[2-hydroxy-3-nonyl]adenine (EHNA), 0 164% Triton X-100 and 1 1 mM dithiothreitol (DTT) DTT was added just before use The assay was performed in the same buffer at 37 °C for 20 min The enzyme activity was measured in the synthetic direction When L-homocysteine was the substrate the enzyme was assayed in quadruplicate at 11 different concentrations in the range from 0-800 μM When D-homocysteine was the substrate the enzyme was assayed in quadruplicate at seven different concentrations in the range from 0-8 mM Adenosine was added in equimolar amounts to L- or D-homocysteine The total volume was 200 μl and it contained the homogenate of ⅛ embryo After incubation protein was precipitated with 12 μl 8 M perchloric acid After 10 min on ice the mixture was centrifuged in an Eppendorf centrifuge for 5 min (13,000 g, 4 °C) and 25 μl 4 M K2HPO4 was added to the supernatant After another 10 min on ice the precipitate was removed by centrifugation and the supernatant was frozen in liquid nitrogen Samples were stored at -80 °C until further analysis SAH was measured by high-performance liquid chromatography (HPLC) and spectrophotometric detection according to DeAbreu *et al* (1982) To calculate enzyme kinetic parameters, the data were converted to a Lineweaver-Burk plot

Measurement of metabolites

For measurement of homocysteine, methionine, SAM and SAH embryos proper and whole conceptuses were thawed, placed on ice, pooled in three series of either 5 whole conceptuses or 9 embryos proper in 300 μl potassium phosphate buffer (pH 6 0) and homogenized by sonication

Homocysteine concentrations were measured with HPLC and fluorometric detection according to Fiskerstrand *et al* (1993) in 70 μl of the homogenate The detection limit was 35 pmol Both inter- and intra-assay CV were <5%

For determination of methionine the homogenates (50 μl) were deproteinized by adding an equal volume of ice-cold sulphosalicylic acid (25% w/v) and were placed on ice After 10 min the samples were centrifuged for 10 min at 3,500 g The supernatant was filtrated through a 0 45 μm filter Methionine concentrations were measured with an automated amino acid analyzer (Pharmacia LKB Alpha-Plus) A 140 μl sample was injected on an cation-exchanged column (24 cm, internal diameter 4 6 mm, particle size 6 μm) After post-column derivatization with *O*-phtaldialdehyde, the eluent was monitored fluorometrically The detection limit was 15 pmol Both inter- and intra-assay CV were <5%

For measurement of SAM and SAH concentrations protein of the homogenates was precipitated by addition of 15 μl ice-cold 50% trichloroacetic acid (TCA) to 150 μl homogenate After 10 min on ice the samples were centrifuged for 2 min at 13,000 g The supernatants were collected and washed three times with two volumes of peroxide-free diethylether to remove TCA The samples were flushed with nitrogen, while on ice, and diluted with aquadest to an end volume of 200 μl SAM and SAH were separated and quantitated by means of HPLC and spectrophotometric detection according to Molloy *et al* (1990)

Chemicals

Homocysteine was prepared freshly by dissolving homocysteinethiolactone in 200 μl 5 N sodium hydroxide and incubation during five minutes at 37 °C The solution was neu-

tralized by adding 400 μl concentrated (×18 3) Tyrode's buffer (pH 7 6) without sodium-chloride and 400 μl 2 5 N HCl and was diluted with 6 3 ml water, which resulted in final concentrations identical to those in Tyrode's buffer This stock solution was further diluted with Tyrode's buffer to the desired concentrations of homocysteine

Homocysteinethiolactones, L-homocystine, L-methionine, L-serine, 5-methylTHF, folinic acid, SAM, SAH and catalase (2,800 IE/mg, prepared from bovine liver) were purchased from Sigma (St Louis, MO), vitamin B12b (OH B12) was obtained from Fluka Chemie (Buchs, Germany), adenosine came from USB (Cleveland, OH), Penicillin G, sodium salt was obtained from Gist/Brocades (Delft, The Netherlands), streptomycinesulfate from Biochemie (Vienna, Austria), D-glucose from Merck (Darmstadt, Germany), EHNA from Burroughs Welcome (Research Triangle Park, NC), and Triton X-100 from Serva Feinbiochemica (Heidelberg, Germany)

Statistical analysis

In the experiments using gestational day 10 conceptuses least square means of TMS and number of dysmorphogenic features/conceptus were calculated by analysis of covariance, taking the number of somite pairs at the beginning of culture as covariant Least square means were compared by Student's *t*-test with Bonferroni correction With a nominal α of 0 05, differences were regarded as statistically significant at $P<0\ 05/k$ where k is the number of comparisons made Since in the experiments with gestational day 9 5 embryos, more than one embryo per bottle was cultured, analysis of covariance could not be applied and analysis of variance was used instead Frequencies of specific dysmorphogenic features were tested with Pearson's chi-square test Unexpected distribution of frequencies was regarded as statistically significant at $P<0\ 01$ If 50% or more of the cells had expected counts of less than five, the test was regarded not valid Student's *t*-test with Cochran's approximation was used to test differences in the levels of metabolites measured Correlations between metabolite levels were tested by calculating Pearson's correlation coefficient in a linear regression model

Results

Prevention of neural tube defects by L-homocysteine, L-homocystine and L-methionine in gestational day 9 5 rat embryos

Rather large amounts of L-homocysteine had to be added to the culture of gestational day 10 rat embryos to elicit embryotoxicity and no NTDs were observed Gestational day 9 5 rat embryos are generally considered to be more sensitive and the neurulation process is still at the neural plate stage We therefore cultured gestational day 9 5 embryos in the presence of L-homocysteine, L-homocystine, L-methionine and folinic acid Despite addition of rat serum to the human serum control embryos developed poorly The dysmorphogenic features in controls were open prosencephalon, open mesencephalon, open rhombencephalon, haemorrhages in the head region and deformed optic primordia However addition of L-homocysteine (1 or 2 mM), L-homocystine (2 mM) or L-methionine (1 mM) resulted in well developed embryos after 48 hr of culture

		N†	Dead	TMS‡	Dysmorphogenic features¶
Control		**29**	2	37 5±0 8	4 4±0 4
L-homocysteine	**300 µM**	**12**		38 3±1 2	3 8±0 6
	1 mM	**12**		46.9±1 2*	0 2±0 6*
	2 mM	**11**		46 8±1 3*	0 2±0 6*
	4 mM	**7**	7		
L-homocystine	**2 mM**	**12**		44 1±1 2*	0 8±0 6*
L-methionine	**30 µM**	**13**		40 4±1 2	3 0±0 6
	100 µM	**11**		44 7±1 3*	1 5±0 6*
	300 µM	**11**		43 4±1 3*	1 3±0 6*
	1 mM	**13**		45 8±1 2*	0 3±0 6*
Folinic acid	**1 mM**	**12**		39 8±1 2	3 9±0 6*

Table 9.1 Effect of L-methionine, L-homocysteine, L-homocystine, and folinic acid on the culture of gestational day 9 5 rat embryos Values shown are least square means±SEM

†No of embryos

‡Total morphological score

¶No of dysmorphogenic features/conceptus

Lower concentrations of L-homocysteine did not improve embryonic development, but TMS increased significantly and number of dysmorphogenic features/conceptus decreased significantly when 100 or 300 µM L-methionine was added. Addition of 4 mM L-homocysteine to the culture killed all gestational day 9.5 embryos during culture. The addition of the folate derivative folinic acid did not improve embryonic development (Table 9.1).

Embryotoxicity of L-homocysteine and its attenuation by 5-methylTHF, L-serine and vitamin B12 in gestational day 10 rat embryos.

The addition of 4 or 6 mM L-homocysteine to the culture of gestational day 10 rat conceptuses resulted in high frequencies of dysmorphogenic features (Table 9.2) and a reduced TMS (Tables 9.3, 9.4 and 9.7)

To test whether the embryotoxicity of L-homocysteine could be attenuated by stimulation of its metabolism 5-methylTHF, L-serine and vitamin B12 were added to the culture. The addition of either 100 µM 5-methylTHF or 6 mM L-

			L-Homocysteine	
		Controls (n=81)	4 mM (n=29)	6 mM (n=56)
Yolksac circulation	Defective	1 (1)	8 (28)	15 (27)*
	Small† yolksac vessels	1 (1)	2 (7)	11 (20)*
Allantois	Not fused with chorion	20 (25)	9 (31)	17 (31)
	Small† or avascular	2 (2)	0 (0)	6 (11)
Flexion	Incomplete	2 (2)	9 (31)	14 (25)*
	Absent	0 (0)	1 (3)	0 (0)
	Inverted tail (positioned at wrong side of head)	10 (12)	3 (10)	10 (18)
Heart	Delayed cardiac tube formation	2 (2)	0 (0)	4 (7)
	Wide† pericardial sac, filled with fluid	0 (0)	0 (0)	4 (7)
Caudal neural tube	Irregular dorsal midline	0 (0)	3 (10)	1 (2)
	Aplasia	0 (0)	2 (7)	9 (16)*
	Kinky	1 (1)	6 (21)	24 (43)*
	Curving more extreme	0 (0)	2 (7)	7 (13)
Rhombencephalon	Open	1 (1)	1 (3)	0 (0)
	Large† or transparent	0 (0)	1 (3)	17 (30)*
Mesencephalon	Open	0 (0)	2 (7)	0 (0)
Prosencephalon	Open	0 (0)	2 (7)	0 (0)
	Small†	0 (0)	1 (3)	15 (27)*
Optic primordium	Deformed	1 (1)	2 (7)	18 (32)*
Branchial bars	One pair or more missing	0 (0)	0 (0)	7 (13)
Maxillary process	Small†	2 (2)	4 (14)	27 (48)*
Mandibular processes	Unapproached†	0 (0)	1 (3)	0 (0)
Other head	Irregular wavy suture line of neural folds	2 (2)	0 (0)	0 (0)
	Abnormal craniofacial appearance	2 (2)	1 (3)	1 (2)
	Haemorrhagic areas	2 (2)	5 (17)	5 (9)
Forelimb buds	Small†	0 (0)	3 (10)	5(9)
	Missing	2 (2)	16 (55)	47 (84)*
Somites	Small†	0 (0)	0 (0)	1 (2)
	Irregular	0 (0)	6 (21)	34 (61)*
	Amorphogenesis	2 (2)	6 (21)	32 (57)*
Others	Edema	1 (1)	15 (52)	35 (63)*
	Haemorrhages	5 (6)	8 (28)	16 (29)*
	General growth retardation	4 (5)	12 (42)	35 (63)*
	Embryonic death	0 (0)	1 (3)	1 (2)
	Cell death‡	2 (2)	23 (79)	53 (95)*

Table 9.2 Dysmorphogenic features of gestational day 10 conceptuses cultured with L-homocysteine. Cumulative data from 10 separate experiments Values shown are no of conceptuses Percentages are in parentheses *P<0 01 in Chi-square test †As compared to the normal situation ‡Visible as opaque regions or spots at places where these normally are not present.

	N†	TMS‡	Dysmorphogenic features¶
Control	**18**	42.8±0 7*	0 4±0.2*
6 mM L-homocysteine	**12**	32.2±1 5§	10 2±0.2§
6 mM L-homocysteine + 100 µM 5-methylTHF	**11**	36.5±1 4*§	7 4±1 4§
6 mM L-homocysteine + 6 mM L-serine	**10**	35 9 1 5§	8.0±1.4§
6 mM L-homocysteine + 10 µM B12	**11**	42 2±0 8*	2 1±0 8*
6 mM L-homocysteine + 100 µM 5-methylTHF + 6 mM L-serine	**12**	35 9±1 5*§	7.2±1 2§
6 mM L-homocysteine + 100 µM 5-methylTHF + 10 µM B12	**11**	42 2±0 6*	1.4±0 5§
6 mM L-homocysteine + 6 mM L-serine + 10 µM B12	**11**	43.2±0 8*	0 7±0 3§
6 mM L-homocysteine + 100 µM 5-methylTHF + 6 mM L-serine + 10 µM B12	**12**	43 3±0.6*	0 5±0 2*

Table 9.3 Attenuation of L-homocysteine embryotoxicity in gestational day 10 rat embryos by 5-methylTHF, L-serine and vitamin B12 Values shown are least square means ±SEM.

†No. of embryos

‡Total morphological score

¶No. of dysmorphogenic features/conceptus

*P<0 00625 in Student's *t*-test when compared with embryos exposed to L-homocysteine alone.

§ P<0 00625 in Student's *t*-test when compared with controls

serine attenuated the embryotoxicity of L-homocysteine. The TMS increased (P-values 0.0013 and 0.0065, respectively) and the dysmorphogenic features/ conceptus decreased (P-values 0.02 and 0.08, respectively) relatively to those embryos that were exposed to L-homocysteine alone. More striking was the effect of the addition of vitamin B12. Both morphological parameters of the embryos grown in the presence vitamin B12 were no longer different from controls (Table 9.3, Fig. 9.2).

Homocysteine oxidation and attenuation of L-homocysteine embryotoxicity in gestational day 10 rat embryos by vitamin B12, Cu(II)SO4 but not by catalase.

Since vitamin B12 can act as an transition metal ion in the catalysis of L-homo-

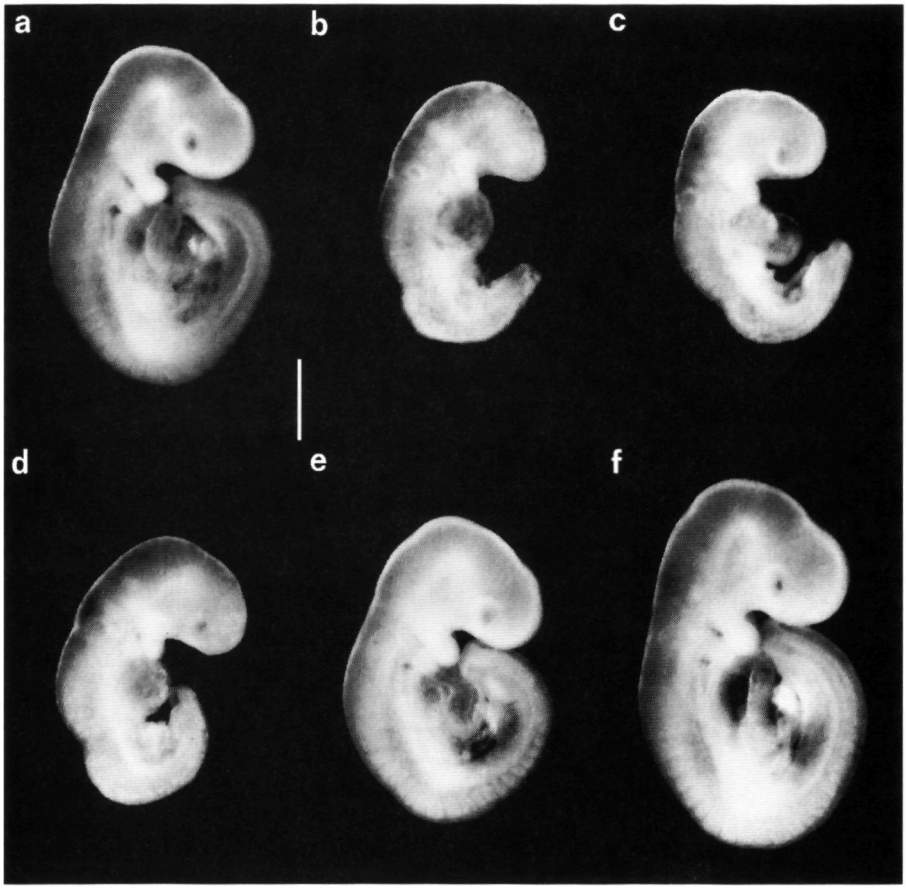

Fig. 9.2. Attenuation of L-homocysteine toxicity in gestational day 10 rat embryos*in vitro* by 5-methylTHF, L-serine and vitamin B12. Embryos were treated as follows. **a**: Control, **b**: 6 mM L-homocysteine, **c**: 6 mM L-homocysteine + 6 mM L-serine, **d**: 6 mM L-homocysteine + 100 μM 5-methylTHF, **e**: 6 mM L-homocysteine + 10 μM vitamin B12 and **f**: 6 mM L-homo-cysteine + 6 mM L-serine + 100 μM 5-methylTHF + 10 μM vitamin B12. Bar indicates 1 mm.

cysteine to L-homocystine, we investigated if the observed effect of vitamin B12 could be matched by the addition of another transition metal ion, Cu^{2+}. Both vitamin B12 and Cu(II)SO4 increased the rate of homocysteine oxidation in the medium under whole embryo culture conditions (Fig. 9.3). The pseudo-first-order rate constant of homocysteine oxidation without further additions was $7.67x10^{-5}$ sec^{-1}, whereas these rate constants increased, when 10 μM vitamin B12 or 10 μM Cu(II)SO4 were added, to $1.77x10^{-4}$ sec^{-1} and $9.78x10^{-5}$ sec^{-1}, respectively. The half-lives of homocysteine in the medium corresponding to these rates of oxidation were 150, 65 and 118 min, respectively. Addition of Cu(II)SO4, like vitamin B12, allowed normal embryonic development, as assessed by the TMS and the number of dysmorphogenic features/conceptus (Table 9.4). H2O2 is proposed as a mediator in the toxicity of L-homocysteine to endothelial cells (Starkebaum and Harlan, 1986). To investigate if such a mechanism is applicable to L-homocysteine embryotoxicity, catalase was added to the culture as well. It was found that catalase had no significant attenuating effect on L-homocysteine embryotoxicity (Table 9.4).

Levels of homocysteine, methionine, S-adenosylmethionine and S-adenosylhomocysteine in gestational day 11 and gestational day 11.5 rat embryos.

An alternative hypothesis for the mechanism of L-homocysteine embryotoxicity could be an imbalance in homocysteine/methionine metabolism caused by elevated embryonic homocysteine concentrations. To investigate this hypothesis, we first compared physiological metabolite levels in gestational day 11.5 rat conceptuses immediately frozen after explantation (*in vivo*) and conceptuses cultured for 2 hr (*in vitro*). It was found that culturing the conceptuses did not change methionine, SAH and homocysteine concentrations significantly. However SAM increased twofold (Table 9.5). Homocysteine and methionine content of whole conceptuses were five- to tenfold of those found in the embryos proper, whereas SAM and SAH content of whole conceptuses were only twice that found in embryos proper. Since the volume of the whole conceptus is roughly ten

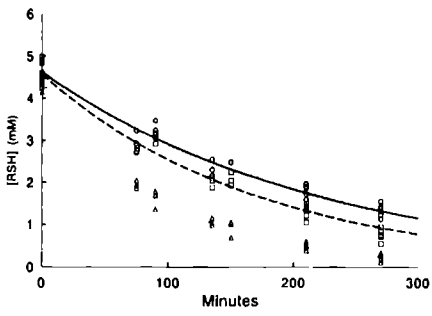

Fig. 9.3. Oxidation of homocysteine under gestational day 10 embryo culture conditions (oxygen concentration 5%) o—o, homocysteine without further additions, Δ···Δ, homo-cysteine in the presence of 10 μM vitamin B12 and □---□, homocysteine in the presence of 10 μM Cu(II)SO4 RSH, free thiol concentration

	N†	TMS‡	Dysmorphogenic features¶
Control	7	42 8±0 3*	0 5±0 1*
6 mM L-homocysteine	7	33 1±0 6§	9 4±0 3§
6 mM L-homocysteine + 10 μM B_{12}	7	42 6±0 5*	1 2±0 1*
6 mM L-homocysteine + Cu(II)SO_4	8	42 6±0 2*	1 4±0 2*
6 mM L-homocysteine + catalase (500 IU/ml)	6	37 0±0 5§	8 8±0 7§

Table 9.4 Effect of vitamin B_{12}, Cu(II)SO_4, and catalase on L-homocysteine embryotoxicity in gestational day 10 rat embryos Values shown are least square means ±SEM
†No of embryos
‡Total morphological score
¶No of dysmorphogenic features/conceptus
*P<0 0125 in Student's *t*-test when compared with embryos exposed to L-homocysteine alone
§ P<0 0125 in Student's *t*-test when compared with controls

	In vivo		In vitro	
	Embryo proper	**Embryo + yolksac**	**Embryo proper**	**Embryo + yolksac**
Homocysteine	0 054±0 016	0 35±0 061	0 052±0 018	0 50±0 26
Methionine	2 3±0 48	17±0 51	3 1±0 77	19±2 3
S-adenosylmethionine	0 27±0 051	0 54±0 046	0 59±0 13	1 2±0 16*
S-adenosylhomocysteine	0 013±0 001	0 048±0 012	0 013±0 001	0 024±0 004

Table 9 5 Levels of methionine, homocysteine, *S*-adenosylmethionine, and *S*-adenosylhomocysteine in gestational day 11 5 rat embryos *in vivo* and after 2 hr of culture (nmol/embryo) Values shown are means based on three different pools of 9 embryos or 5 embryos + yolksac ± SEM *P<0 05 in Student's *t*-test when compared to *in vivo* embryos

times that of the embryo proper, this indicates that homocysteine and methionine are present intracellularly and in extraembryonic fluids at comparable concentrations, whereas SAM and SAH seem to be largely confined to the cellular compartment.

The addition of 3 mM L-homocysteine or D-homocysteine to the culture of gestational day 11 embryos increased the homocysteine content of the embryos

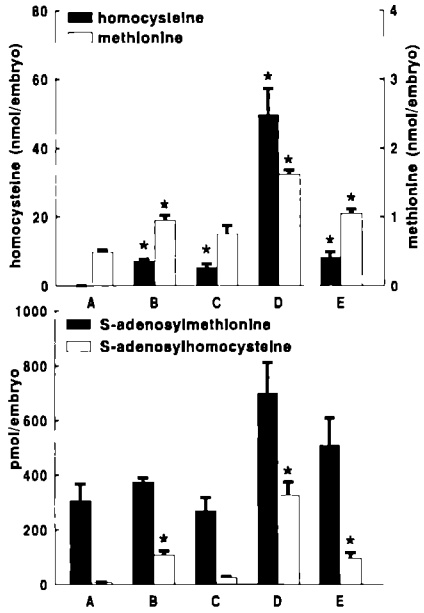

Fig. 9.4 Levels of homocysteine, methionine, S-adenosylhomocysteine and S-adenosylmethionine in gestational day 11 rat embryos after 2 hr of culture in (A) 75% human serum and 25% Tyrode's buffer or the same medium to which (B) 3 mM L-homocysteine, (C) 3 mM D-homocysteine, (D) 3 mM L-homocysteinethiolactone or (E) 3 mM D-homocysteinethiolactone had been added. Values shown are means ± SEM of three pools of nine embryos proper for each group. * = $P<0.05$ when compared with controls (group A) in Student's *t*-test.

proper 180- and 133-fold, respectively (Fig. 9.4). L-homocysteine increased embryonic methionine 1.9-fold and SAH 14-fold. D-homocysteine increased the methionine levels 1.5-fold and SAH only 3-fold. The homocysteinethiolactones increased embryonic homocysteine, methionine and SAH levels even more, and again stereospecific differences were observed. The SAM/SAH ratio, an important determinant for the rate of transmethylation reactions, was 42 in controls. Exposure to 3 mM L-homocysteine reduced it to 3.6. After D-homocysteine exposure it was 11. L- and D-homocysteinethiolactone reduced it to 2.1 and 5.3, respectively.

Stereospecificity of S-adenosylhomocysteine hydrolase

When fitted in a linear regression model embryonic SAH levels and embryonic homocysteine levels correlated well, both after D- and L-homocysteine exposure, however the regression coefficients differed markedly. D-homocysteine resulted in a regression coefficient (± SEM) of 0.0044±0.0004 (Pearson correlation coefficient 0.996, $p=0.06$, $n=3$). L-homocysteine resulted in a regression coefficient of 0.012±0.003 (Pearson correlation coefficient 0.83, $p=0.006$, $n=9$). This suggests that the stereospecific embryotoxicity of homocysteine might be caused by a difference in the rate of conversion of homocysteine to SAH by SAH hydrolase. We therefore assayed this enzyme in the synthetic direction with both D- and L-homocysteine as a substrate (Table 9.6). The Km of the enzyme for L-homocysteine is 79 times as low as the Km for D-homocysteine, demonstrating a clear stereospecific preference of the enzyme. The Km for L-homocysteine in embryo homogenate found by us is the same as the one reported for the purified rat liver enzyme (Kajander and Raina, 1981). Substrate inhibition as reported by Duerre and coworkers (Walker and Duerre, 1975;

Substrate	Km† (μM)	Vmax† (nmol•min^{-1}• embryo^{-1})
L-Homocysteine‡	62	5 05
D-Homocysteine¶	4 9×10^{3}	3 3

Table 9.6 Stereospecificity of *S*-adenosylhomocysteine hydrolase. Enzymatic activity assayed for the synthesis of *S*-adenosylhomocysteine †Calculated after conversion of the data to a Lineweaver-Burk plot. ‡Values shown are based on quadruplicate measurement at 11 different concentrations ¶Values shown are based on quadruplicate measurement at seven different concentrations

Hoffman *et al.*, 1979) was only observed with D-homocysteine at the highest concentration (8 mM).

Attenuation of L-homocysteine embryotoxicity in gestational day 10 rat embryos by L-methionine.

Since embryotoxicity of L-homocysteine is possibly caused by a reduction of the SAM/SAH ratio we examined if this embryotoxicity could be attenuated by addition of L-methionine. The addition of high doses of L-methionine (6 and 12 mM) improved the morphology slightly, as compared to the embryos exposed to L-homocysteine alone (P-values for TMS were 0.11 and 0.07, respectively, and those for the number of dysmorphogenic features/conceptus were 0.07 and 0.06, respectively) (Table 9.7).

	N†	TMS‡	Dysmorphogenic features¶
Control	12	45.0±0.8	0.1±0.1
6 mM L-homocysteine	12	34.7±1 2*	10 0±0 9*
6 mM L-homocysteine + 1.5 mM L-methionine	12	34.2±1.2*	10 3±1 0*
6 mM L-homocysteine + 3 mM L-methionine	12	33.4 1 9*	11 4±1 2*
6 mM L-homocysteine + 6 mM L-methionine	12	37 0±1 0*	7 5±0 9*
6 mM L-homocysteine + 12 mM L-methionine	12	37 3±1 1*	7 4±1 2*

Table 9.7 Attenuation of L-homocysteine embryotoxicity in gestational day 10 rat embryos by L-methionine Values shown are least square means ±SEM.
†No of embryos
‡Total morphological score
¶No. of dysmorphogenic features/conceptus.
*P<0 01 in Student's *t*-test when compared with controls

Discussion

Gestational day 9.5 rat embryos developed very well when 1 or 2 mM L-homocysteine was added to the culture, whereas these embryos had an open prosencephalon, an open mesencephalon, an open rhombencephalon, haemorrhages in the head region and deformed optic primordia when L-homocysteine was omitted. These dysmorphogenic features can all be related to the failure of one process, namely closure of the anterior neural tube. This is the first demonstration that L-homocysteine promotes embryonic development of rat embryos *in vitro* when it is added to serum that without supplementation does not support normal embryonic development. L-methionine had the same beneficial effect on gestational day 9.5 rat embryos at even lower concentrations. This finding is in line with reports from other laboratories (Coelho *et al.*, 1989; Coelho and Klein, 1990; Ferrari *et al.*, 1993; Flynn *et al.*, 1987). Coelho and Klein (1990) could not replace methionine by homocysteine to prevent NTDs when gestational day 9.5 rat embryos were cultured in cow serum, but they used only 185 μM. In the gestational day 9.5 embryo culture we tested L-homocystine only at a 2 mM concentration. At this concentration it was just as effective as 1 or 2 mM L-homocysteine in preventing NTDs. This suggests that it may be reduced intracellularly to L-homocysteine. However uptake of the oxidized from will be limited, or its intracellular reduction may be incomplete, since an equimolar amount of homocysteinyl moieties in their reduced form (4 mM L-homocysteine) killed all embryos.

The beneficial effect of L-homocysteine in the culture of gestational day 9.5 rat embryos can be explained in two ways. First, L-homocysteine will be converted to L-methionine and thus supply the embryo with the amino acid that is probably limiting in embryonic development. Second, conversion of L-homocysteine to L-methionine will concomitantly demethylate 5-methylTHF, thus liberating THF for other essential functions such as purine and thymidine synthesis. Our observations that methionine is about 10 times as effective in promoting development of gestational day 9.5 rat embryos as compared to L-homocysteine and that folinic acid did not promote development both favour the first explanation. It is not clear which processes are disturbed by methionine deficiency. Methionine is used for protein synthesis and it is converted to SAM. SAM is the universal methyl group donor in transmethylation reactions, and both reduced DNA methylation (Matsuda and Yasutomi, 1992; Li *et al.*, 1992) and reduced protein methylation (Coelho and Klein, 1990) have been suggested as teratogenic mechanisms. SAM is also a precursor in polyamine synthesis and reduced synthesis of these bioamines may deteriorate embryonic development as well. Finally when SAM levels are reduced the negative feedback on the reduction of N^5,N^{10}-CH2-THF to 5-methylTHF by SAM will be abolished (Shane and Stokstad, 1983). Thus low levels of SAM will divert the folate pool to the forma-

tion of 5-methylTHF. Since homocysteine can only be formed from SAH, the demethylated form of SAM, it is likely that an embryo deficient in methionine will have low homocysteine levels as well. However, methionine synthase, the only enzyme capable of demethylating 5-methylTHF, needs homocysteine as a substrate (Finkelstein, 1990) (Fig. 9.1). Methionine deficiency considered in this way, should therefore more appropriately be regarded as a deficiency in homocysteinyl moieties. Under these conditions not only reactions using SAM as a substrate will be hampered, but folate may be trapped as 5-methylTHF to some extent as well. Nevertheless, in our experiments a possible folate trapping effect of a homocysteinyl deficiency could not be counteracted upon by addition of 1 mM folinic acid.

The observation that the addition of L-homocysteine prevents NTDs in the culture of gestational day 9.5 rat embryos, is not in favour of a previous hypothesis that L-homocysteine may be the teratogen leading to this defect in women with mild hyperhomocysteinemia (Steegers-Theunissen, 1993; Steegers-Theunissen *et al.*, 1993). Blood homocysteine levels in these women were only marginally increased. Furthermore plasma homocysteine is mostly protein-bound as a protein-homocysteine mixed-disulfide (Ueland *et al.*, 1992) and of the free homocysteine only one tenth is in its reduced form (Araki and Sako, 1987). Blood homocysteine levels elevated to a greater degree as compared to healthy controls after an oral methionine load should therefore only be interpreted as a biochemical risk indicator, showing homocysteine-methionine metabolism is not functioning optimally.

High concentrations of L-homocysteine were embryotoxic to both gestational day 10 (1.8 mM and higher, VanAerts *et al.*, 1993a) and gestational day 9.5 (4 mM) rat embryos. The difference in sensitivity may have resulted from a higher rate of uptake in gestational day 10 rat embryos, as at this stage visceral yolk sac circulation is already present. Many dysmorphogenic features are observed in gestational day 10 rat conceptuses affecting many embryonic structures. This may be a reflection of reduced cell growth and increased cell death, since previous histological observations showed reduced mitotic indices of the neural epithelium, reduced cell densities of the mesenchyme adjacent to the neural tube and many apoptotic bodies (VanAerts *et al.*, 1993a). A frequent dysmorphogenic feature in gestational day 10 conceptuses, not related to L-homocysteine exposure, was failure of fusion of the allantois to the chorion (Table 9.2). This was not observed when gestational day 9.5 conceptuses were cultured. It therefore seems to be related to the stage of development at the time of explantation of the conceptuses. Failure of fusion of the allantois had no adverse effect on the development of the embryo, except that in 50% of these embryos the caudal part of the embryo was positioned at the wrong side of the head. This indicates that if fusion fails at this stage of development positioning of the tail occurs randomly.

The embryotoxicity of L-homocysteine in gestational day 10 rat embryos could be attenuated by addition of 5-methylTHF or L-serine. 5-MethylTHF is the co-substrate for methionine synthase, the enzyme remethylating homocysteine to methionine. L-serine in turn is a major one-carbon donor providing the methylene moiety in the formation of N^5,N^{10}-CH_2-THF. After reduction of this folate metabolite, 5-methylTHF is formed once again (Fig. 9.1). The attenuating effect of 5-methylTHF and L-serine on L-homocysteine embryotoxicity could therefore be explained by their stimulation of the remethylation of L-homocysteine to L-methionine, which would result in a lowering of embryonic L-homocysteine levels to a less toxic level and an increase of L-methionine levels.

The prevention of L-homocysteine embryotoxicity by vitamin B12 could theoretically be explained by stimulation of the activity of methionine synthase, which would increase the metabolism of L-homocysteine. However, the vitamin B12 effect was abolished when the concentration of vitamin B12 was decreased to 0.1 μM (data not shown). Physiological concentrations are in the picomolar range and cobalamines, like transition metal ions, in micromolar concentrations are known to catalyze thiol oxidation (Jacobsen *et al.*, 1984; Cavallini *et al.*, 1969). However, the oxidation product of L-homocysteine, L-homocystine, is a less potent embryotoxicant (VanAerts *et al.*, 1993a). It seemed therefore more likely that the vitamin B12 effect was mediated by an oxidative process. This conclusion is further substantiated by the observation that the transition metal ion Cu^{2+}, which also catalyses thiol oxidation, has the same attenuating effect. The observed effect of vitamin B12 also focussed our attention on a theory, which states that the toxicity of homocysteine to endothelial cells is mediated by a Cu^{2+}-catalyzed, H_2O_2 producing oxidation of homocysteine (Starkebaum and Harlan, 1986). However catalase, a H_2O_2 scavenger, had no significant attenuating effect on L-homocysteine embryotoxicity and Cu^{2+} abolishes L-homocysteine embryotoxicity. This suggests that L-homocysteine embryotoxicity is not mediated by a Cu^{2+}-catalyzed, H_2O_2 producing oxidation of homocysteine. On the contrary, the catalysis of the oxidation of L-homocysteine to the less toxic disulfide L-homocystine by vitamin B12 and Cu(II)SO_4 is the most probable explanation for the abolishment of the embryotoxicity of L-homocysteine.

To investigate whether a disturbance of normal homocysteine metabolism by exposure to high concentrations of L-homocysteine could provide an explanation for L-homocysteine embryotoxicity, we exposed gestational day 11 rat embryos to 3 mM L- and D-homocysteine and their thiolactones and measured embryonic homocysteine, methionine, SAM and SAH levels. Exposure to L-homocysteine increased embryonic homocysteine concentrations dramatically, indicating that L-homocysteine was readily taken up. SAH concentrations were most probably increased by SAH hydrolase as it will convert part of the homocysteine to SAH. Although under physiological conditions this enzyme hydrolyses SAH, it will synthesize SAH in the presence of high concentrations of homo-

cysteine, since the reaction is reversible and synthesis is energetically even more favourable (Ueland, 1982). Because SAM levels did not change, the elevation of SAH resulted in a dramatic lowering of the SAM/SAH ratio. A SAM/SAH ratio lower than 4 will inhibit many transmethylation reactions up to 60% (Cantoni *et al.*, 1979). Thus, when embryonic L-homocysteine levels are elevated too much this would lower the SAM/SAH ratio below a critical level and thereby inhibit crucial transmethylation reactions. Reduction of the SAM/SAH ratio as a mechanism for L-homocysteine embryotoxicity may also provide an explanation for the attenuating effect of L-methionine on L-homocysteine embryotoxicity. When methionine concentrations are high SAM synthesis will be increased. Higher SAM levels will increase the SAM/SAH ratio and thereby counteract on the reduction of the SAM/SAH ratio by elevated SAH levels. Nevertheless, a low SAM/SAH ratio might not be the only possible way in which increased SAH formation could be toxic. Since adenosine is the co-substrate for SAH hydrolase, increased SAH formation by this enzyme may lead to reduction of cellular adenosine concentrations, which also may cause toxicity to the embryo.

Exposure of gestational day 11 embryos to 3 mM D-homocysteine increased embryonic homocysteine levels to almost the same extent as L-homocysteine did. However, due to the high Km of SAH hydrolase for D-homocysteine, D-homocysteine increased SAH levels to a much lesser extent than L-homocysteine. This supports our hypothesis that increased SAH formation is the first step in the embryotoxic mechanism of L-homocysteine, since D-homocysteine was shown not to be embryotoxic at a concentration where L-homocysteine was very embryotoxic (VanAerts *et al.*, 1993a).

The homocysteinethiolactones were included in our experiments since they have been used as a test compound in other studies on the toxicity of homocysteine (Dudman and Wilcken, 1982; Ueland *et al.*, 1992) and it has the advantage that it cannot oxidize to a disulfide form. However, the biological relevance may be low, since reports that homocysteinethiolactone is formed in the body could not be confirmed by others (Ueland *et al.*, 1992). Nevertheless, increase of both methionine and SAH after exposure to homocysteinethiolactone indicates that the thiolactone ring can be hydrolysed biologically. Since thiolactone esterase, the enzyme performing this reaction, is absent in human plasma (Dudman and Wilcken, 1982) this enzyme is apparently present in the conceptus. The large difference in embryonic homocysteine levels after D- or L-homocysteine-thiolactone exposure probably reflects the stereospecific preference of this enzyme (Dudman and Wilcken, 1982). The lowering of the SAM/SAH ratio caused by the thiolactones was comparable with that observed after L-homocysteine exposure. Therefore our previous observation that both thiolactones are embryotoxic (VanAerts *et al.*, 1993a) may also be explained by increased SAH formation, leading to reduced SAM/SAH ratios and consequently inhibition of transmethylation reactions. However, other effects, especially thiolactone-de-

pendent acylation of proteins, may have attributed to the toxicity of the homocysteinethiolactones as well (Ueland *et al.*, 1992).

Based on the evidence presented in this paper, we propose that L-homocysteine embryotoxicity, as observed in our experiments, is not mediated by an oxidative process, but rather is initiated by increased SAH formation by SAH hydrolase leading to reduced SAM/SAH ratios and consequently inhibition of transmethylation reactions. The beneficial effect of L-homocysteine on the development of gestational day 9.5 embryos and the fact that only very high concentrations of L-homocysteine caused embryotoxicity makes it very unlikely that L-homocysteine is the teratogen causing NTDs in children from mild hyperhomocysteinemic women. Our results suggest that methionine plays an important role in the neurulation process. Future research should therefore be directed to the elucidation of the role of methionine in neurulation.

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Development Of Methionine Synthase, Cystathionine-ß-Synthase And S-Adenosylhomocysteine Hydrolase During Gestation In The Rat.

10

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Abstract The developmental onset of three homocysteine metabolizing enzymes in the rat conceptus was investigated. Cystathionine-ß-synthase and methionine synthase were assayed from day 10 to day 20 of gestation in decidual and placental tissue, from day 10 to day 12 of gestation in embryonic tissue, from day 14 to day 20 of gestation in fetal liver and from day 14 to day 20 of gestation in fetal tissue without liver. On each day, material was obtained from at least four conceptuses from two dams. S-adenosylhomocysteine hydrolase was assayed in neurulating conceptuses in decidual tissue, parietal yolksac plus ectoplacental cone, visceral yolksac plus amnion and embryo proper. Conceptuses were pooled from seven (day 9.5 of gestation) or three (days 10.5 and 11.5 of gestation) dams. In embryonic and fetal tissue cystathionine-ß-synthase first occurred in fetal liver During the organogenic phase it was present only in decidual tissue. Methionine synthase was present in all tissues from the first gestational day investigated and S-adenosyl-homocysteine hydrolase was present in all tissues throughout the neurulating period. Our results indicate that the homocysteine-methionine cycle, which is crucial to trans-methylation reactions, is functional during the neurulating period in embryonic tissue. Owing to the absence of cystathionine-ß-synthase at this stage of development in embryonic tissue, the homocysteinyl moiety is conserved in the homocysteine-methionine cycle.

Introduction

A disturbance of methionine-homocysteine metabolism may play a role in the aetiology of neural tube defects, and also in other obstetrical problems, such as recurrent spontaneous abortion and abruptio placentae (Schorah *et al.*, 1993; Steegers-Theunissen, 1993; Steegers-Theunissen *et al.*, 1991, 1992; Wouters *et al.*, 1993).

L-Homocysteine can be metabolised by several enzymes (Fig. 10.1). Methionine

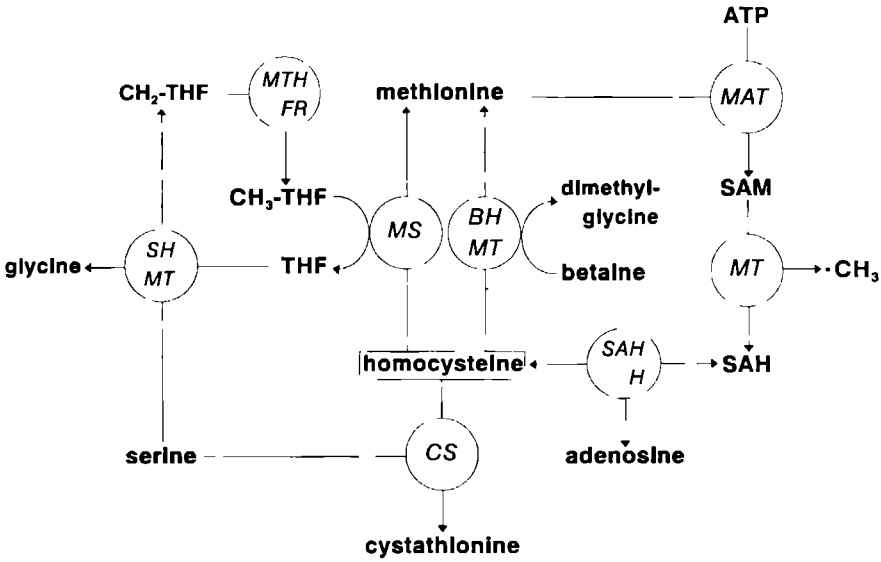

Fig. 10.1 Homocysteine and methionine metabolism and its relation to folate metabolism *BHMT* betaine-homocysteine methyltransferase, *CS* cystathionine-ß-synthase, *MAT* methionine-adenosyl transferase, *MS* methionine synthase, *MT.* various methyltransferases, *MTHFR* methylenetetrahydrofolate reductase, *SAHH* S-adenosylhomocysteine hydrolase, *SHMT* serinehydroxymethyltransferase, SAM S-adenosylmethionine, SAH S-adenosylhomocysteine, THF tetrahydrofolate, CH2-THF N^5,N^{10}-methylenetetrahydrofolate, CH3-THF N^5-methyltetrahydrofolate

synthase (N^5-methyltetrahydrofolate-L-glutamate:L-homocysteine S-methyltransferase, EC 2.1.1.13) remethylates L-homocysteine to L-methionine. In this way the homocysteinyl moiety is conserved in methionine-homocysteine metabolism. At the same time methionine synthase is the only enzyme in mammals that can demethylate N^5-methyltetrahydrofolate (5-methylTHF). Since 5-methylTHF is the major circulating form of folate, methionine synthase is the key enzyme to their active reduced folate supply, which is essential in one-carbon metabolism, and purine and pyrimidine synthesis. Therefore methionine synthase is considered to be ubiquitous in mammals (Shane and Stokstad, 1983). During gestational development methionine synthase activity seems to progressively decline in several species (Gaull *et al.*, 1973; Sternowsky *et al.*, 1976). However, a developmental study, including the organogenic phase, has never been published.

Betaine L-homocysteine S-methyltransferase (EC 2 1 2 5) can also remethylate L-homocysteine to L-methionine Betaine L-homocysteine S-methyltransferase activity is found in liver tissue of all mammals investigated and also occurs in kidney tissue (Ericson, 1960, Finkelstein *et al*, 1971) In humans betaine L-homocysteine S-methyltransferase activity is also present in both fetal and adult brain tissue, although activity is low (Gaull *et al*, 1973) Since betaine L-homocysteine S-methyltransferase is a typical liver enzyme, and the liver is not present at the time of neurulation, the period under investigation, no attempt was made in this study to measure its activity in rat embryonic and fetal tissue

Cystathionine-ß-synthase (EC 4 2 1 22) condenses L-serine with L-homocysteine to L-cystathionine This is an irreversible step in the transsulfuration pathway, the main pathway for methionine and homocysteine catabolism Generally cystathionine-ß-synthase activity increases during fetal development, however, the developmental onset is unknown, and may differ between species (Volpe and Laster, 1970, 1972, Sturman *et al*, 1976, Rassin *et al*, 1981, Gaull *et al* 1972)

S-adenosylhomocysteine (SAH) hydrolase (EC 3 3 1 1) is the only source of L-homocysteine in mammals as it hydrolyses SAH to L-homocysteine and adenosine However, when L-homocysteine levels are high, SAH hydrolase will synthesize SAH, as the reaction is reversible and SAH synthesis is energetically more favourable (Ueland, 1982) Since SAH accumulation will inhibit crucial transmethylation reactions, in mammals, SAH hydrolase is assumed to be ubiquitous as well However, developmental studies have not been published

The objective of this study was to investigate the gestational development of methionine synthase, cystathionine-ß-synthase and SAH hydrolase in the rat conceptus to provide a better understanding of the possible relationship between methionine-homocysteine metabolism and the aetiology of neural tube defects

Materials and methods

Chemicals

Bovine serum albumin (BSA), 5-methylTHF, complete o-phtaldialdehyde reagent solution, Coomassie Brilliant Blue G, dithiothreitol (DTT), L-homocysteinethiolactone, L-serine, L-cystathionine, S-adenosylhomocysteine (SAH), S-adenosylmethionine (SAM), lubrol PX and pyridoxal phosphate were purchased from Sigma (St Louis, MO) Ascorbic acid and 2-mercaptoethanol were purchased from Merck (Darmstadt Germany) T61 (containing 20% (w/v) embutramide, 5% (w/v) mebezoniumiodide and 0 5% (w/v) tetracaine hydrochloride in water) was obtained from Hoechst Veterinar GmbH (Munich, Germany), hydroxycobalamin (vitamin B12b) from Fluka Chemie (Buchs, Germany), [U ^{14}C]-L-serine (specific activity 166 mCi mmol) from DuPont de Nemours (Dreieich, Germany), erythro-9[2-hydroxy-3-nonyl]adenine (EHNA) from Burroughs Welcome (Research Triangle Park, NC), adenosine from USB (Cleveland, OH) and Triton X-100

from Serva Feinbiochemica (Heidelberg, Germany). All solutions were made in tap water, demineralised by a Milli-RO 10TS system from Millipore Corporation (Bedford, MA), except for the HPLC buffers, which were prepared with demineralised water additionally filtered by a Nanopure system from Sybron/Barnstaedt (De Bugue, IA). L-Homocysteine was prepared from L-homocysteinethiolactone by alkaline hydrolysis (5 N NaOH, 5 min, 37 °C). The solution was neutralized with HCl and diluted with the appropriate assay buffer.

Animals and dissection of tissues

Random bred Cpb:WU (Wistar) rats were housed in pairs or groups of three in cages and allowed to eat (MRH-TM pellets, Hope Farms B.V., Woerden, The Netherlands) and drink (tap water) *ad libitum*. For animals from which material was obtained on days 10, 11, 12, 14, 16, 18 or 20 of gestation, lights were on from 12:00 h until 00:00 h. On day 0, females (11-26 wk old, 200-300 g) were brought together with males (1:1) from 09:00 h until 12:00 h. Animals from which material was obtained on days 9 5, 10.5 or 11.5 of gestation, were housed under the same conditions, except for the light regimen and the time of mating, which were shifted by 12 hr

When the conceptuses had reached the gestational age desired, the dams were killed by an intracardial injection of 0.4 ml T61. The uterus was removed and the conceptuses were explanted and placed in Hank's balanced salt solution. Conceptuses at day 10, 11 or 12 of gestation were dissected into (i) embryo proper and (ii) decidua. Extraembryonic membranes (amnion, visceral and parietal yolk sac and ectoplacental cone) were discarded. Conceptuses at day 14, 16, 18 or 20 of gestation were dissected into (i) fetal liver, (ii) rest of fetus and (iii) placenta plus extraembryonic membranes plus residual decidual tissue. Conceptuses at day 9.5 of gestation were dissected into (i) embryo proper plus amnion plus visceral yolksac, (ii) parietal yolksac plus ectoplacental cone and (iii) decidua. Conceptuses at day 10.5 or 11.5 of gestation were dissected into (i) embryo proper, (ii) amnion plus visceral yolksac, (iii) parietal yolksac plus ectoplacental cone and (iv) decidua. For reference, maternal liver (day 10 of gestation) was used. After dissection, tissues were immediately frozen in liquid nitrogen and stored at -80 °C until further treatment.

Enzyme assays

Protein concentrations were assayed using Coomassie Brilliant Blue G according to Bradford (1965). BSA was used as a standard. All samples were analyzed in triplicate

The methionine synthase and the cystathionine-ß-synthase assays were performed with all tissues obtained from dams on day 10, 11, 12, 14, 16, 18 or 20 of gestation. At each stage, material was obtained from two animals and at least two conceptuses from each animal were assayed in duplicate The methionine synthase assay and measurement of the *o*-phtaldialdehyde derived methionine by HPLC and fluorometric detection were performed according to Garras *et al.* (1991).

The cystathionine-ß-synthase assay was performed according to Fowler *et al.* (1978), except that tissues were homogenized (1:10, w/v) in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% (v/v) lubrol PX and the assay buffer (final volume 100 μl) contained 50 μl homogenate, 25 mM potassium phosphate, 0.05% (v/v) lubrol PX, 108 mM TRIS-HCl (pH 8.6), 8 mM L-serine (final specific activity 1563 μCi mmol^{-1}

[U-^{14}C]-L-serine), 1 M pyridoxal phosphate, 1 5 mM DTT and 15 mM L-homocysteine L-Homocysteine stock solution (7 5 μl) was added to the incubation mixture after a preincubation of 5 min at 37 °C

The S-adenosylhomocysteine hydrolase assay was performed with all tissues obtained on day 9 5 (7 rats, 75 conceptuses), 10 5 (3 rats, 32 conceptuses) or 11 5 (3 rats, 40 conceptuses) of gestation Material from each rat was pooled separately, except for day 9 5 of gestation when material from 2 or 3 rats was used for each pool Thus for each tissue and each gestational stage, tissues were assayed in triplicate Tissues were sonicated at a protein concentration of approximately 10 mg ml ^{1} in ice-cold 22 5 mM potassium phosphate buffer (pH 7 0) containing 1 mM Na2EDTA·2H2O, 20 μM EHNA, 1 64 ml Triton X-100 l ^{1} and 1 1 mM DTT The assay was performed at 37 °C for 20 min in the same buffer (10 μl homogenate in a final volume of 200 μl) additionally containing 0 5 mM L-homocysteine and 0 5 mM adenosine After the incubation protein was precipitated with 12 μl 8 M perchloric acid After 10 min on ice, the mixture was centrifuged in a Eppendorf centrifuge for 5 min (13,000 g, 4 °C) and 25 μl 4 M dipotassium hydrogenphosphate was added to the supernatant After another 10 min on ice the precipitate was removed by centrifugation and the supernatant was frozen in liquid nitrogen Samples were stored at -80 °C until further analysis SAH was measured in duplicate by using HPLC and spectrophotometric detection according to DeAbreu *et al* (1982) All enzyme activities were expressed in μU (pmol product formed min ^{1} mg ^{1} protein)

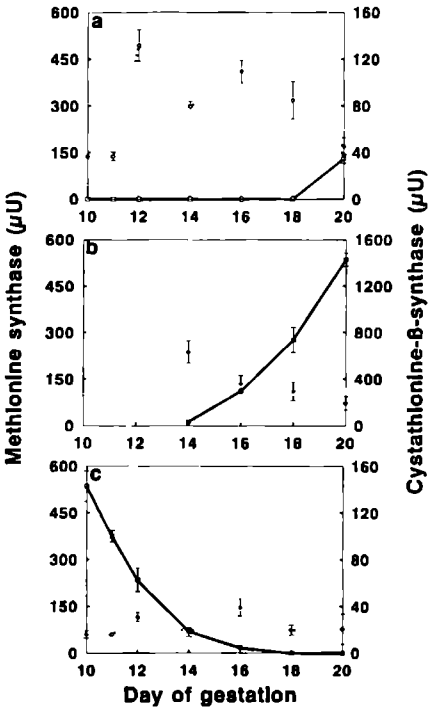

Fig 10.2 Methionine synthase activity (---) and cystathionine-ß synthase activity (___) in (a) embryonic and fetal tissue without liver, (b) fetal liver and (c) decidual and placental tissue during the second half of gestation in rats Each point represents the mean of four conceptuses taken from two dams Error bars represent SEM

Statistical analysis

Linear regression analysis was used to test whether methionine synthase activity or cystathionine-ß-synthase activity increased or decreased during gestation A decrease or increase was regarded as significant if $P<0\ 05$ for the null-hypothesis that the regression coefficient equalled zero Differences in enzyme activity between gestational days and differences in SAH hydrolase activity between tissues for each gestational day were tested using Student's *t*-test with Bonferroni correction With a nominal α of 0 05, differences were regarded as statistically significant at $P<0\ 05\ k^{-1}$, where k is the number of comparisons made All differences, increases, and decreases, in enzyme activities mentioned in the results

section met the degree of statistical significance as mentioned above.

Results

Methionine synthase was present in all tissues investigated. The highest specific activity was found in embryonic tissue on day 12 of gestation. On days 10 and 11 of gestation it was lower and from day 12 onward the activity decreased at an average rate of 33 μU day^{-1}. Methionine synthase specific activity of maternal liver was 180 μU, which was comparable to the average methionine synthase specific activity of fetal liver. Methionine synthase activity of the fetal liver decreased during gestational development at a rate of 26 μU day^{-1}. The lowest methionine synthase specific activity was found in decidual and placental tissue, and here it did not change significantly during gestation. (Fig. 10.2).

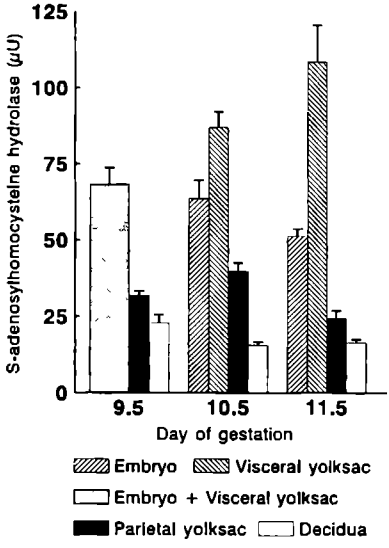

Fig. 10.3 S-adenosylhomocysteine hydrolase activity in neurulating rat conceptuses. Enzyme activity was measured in the direction of synthesis. Conceptuses were separated into embryo proper, visceral yolksac (+ amnion), parietal yolksac (+ ectoplacental cone) and decidua, except for embryos from day 9.5 of gestation, in which embryos and visceral yolksac were pooled together. Bars and error bars represent the mean and SEM of three pools of conceptuses.

During gestation, cystathionine-ß-synthase activity was not present in embryonic tissue. In this organogenic phase of development, it was present only in the surrounding decidual or placental tissue. In the latter tissue, it decreased during gestation at a rate of 15 μU day^{-1} and was virtually absent at day 16 of gestation. In fetal tissue, activity first occurred in the liver, where it was present from the first day (day 14) that it was measured, although initially activity was low. Cystathionine-ß-synthase specific activity of fetal liver increased during the last trimester of gestation, at a rate of 235 μU day^{-1} and reached, on day 20 of gestation, 68% of cystathionine-ß-synthase specific activity of maternal liver, which was 2100 μU. In fetal tissue without liver, cystathionine-ß-synthase activity occurred only on days 18 and 20 of gestation (Fig. 10.2).

SAH hydrolase was present in neurulating rat conceptuses on all days investigated (days 9.5 to 11.5 of gestation), both in the embryos proper and in the surrounding tissues. However, specific activities differed markedly between tissues (Fig. 10.3). On day 9.5 of gesta-

tion, SAH hydrolase specific activity was greater in embryonic plus visceral yolk-sac tissue than it was in decidual or parietal yolksac tissue. On day 10.5 of gestation, the investigated tissues could be ordered in sequence of increasing SAH hydrolase activity as follows: decidua<parietal yolksac<embryo<visceral yolksac. On day 11.5 of gestation, SAH hydrolase activity was greater in visceral yolksac tissue than it was in any other tissue. In addition, embryonic SAH hydrolase activity was greater than decidual SAH hydrolase activity. Interday differences in SAH hydrolase activity were limited. Only SAH hydrolase activity in parietal yolksac tissue on day 11.5 of gestation was lower as compared with that on day 10.5 of gestation. Maternal liver SAH hydrolase specific activity (on day 10 of gestation) was 262±48 (mean±SEM) μU.

Discussion

The data on the developmental onset of cystathionine-ß-synthase in rat fetuses extend those of Volpe and Laster (1972) and show that this enzyme first appears in the fetal liver and that, before this organ is formed, cystathionine-ß-synthase activity is present only in the surrounding decidual and placental tissue. The presence of methionine synthase activity in rat embryos and fetal liver (Hansen and Billings, 1986; Baden *et al.*, 1984; 1987) is confirmed; however, owing to the decrease of methionine synthase activity of fetal liver towards the end of gestation, it appears that the observation from Baden *et al.* (1984; 1987) that fetal liver activity is 50-65% of maternal liver activity is valid only for fetal liver at the end of gestation. The activity of methionine synthase in embryonic and fetal tissues from day 12 to day 18 of gestation was higher relative to maternal liver activity, which is in contrast to the results from Hansen and Billings (1985) that, at day 14 of gestation, activity in embryonic and fetal tissues was the same as the activity of maternal liver. The observations presented here extend those of Hansen and Billings (1985) and, for the first time, demonstrate that methionine synthase activity is present in neurulating rat embryos. In studies on the teratogenicity of nitrous oxide, a methionine synthase inhibitor, the presence of methionine synthase in neurulating embryos was only presumed (Baden *et al.*, 1983; Baden and Fujinaga, 1991; Fujinaga and Baden, 1994).

This is also the first report to demonstrate SAH hydrolase activity in neurulating rat embryos. The higher activity of this enzyme in embryonic and visceral yolksac tissue relative to the activity in the surrounding parietal yolksac and decidua indicates that SAH metabolism occurs mainly in the embryonic and visceral yolksac compartments. The presence of SAH hydrolase and methionine synthase, and the absence of cystathionine-ß-synthase, in the embryo proper indicates that the homocysteine-methionine cycle is functional at this stage of development, but that there is no diversion into the transsulfuration pathway in

the embryo. It is calculated that in adult rat liver tissue about half of the homocysteine is transsulfurated, whereas the other half is remethylated to methionine (Finkelstein, 1990; Finkelstein and Martin, 1986). The proportion of homocysteine that will be remethylated can be increased or decreased depending on the need for SAM. The conservation of the homocysteinyl moiety in the homocysteine-methionine cycle as a result of the absence of cystathionine-ß-synthase in the neurulating embryo emphasizes the importance of this cycle in embryonic development at this stage. The relevance of this notion is also supported by the work of Klein and coworkers (Coelho *et al.*, 1989; Coelho and Klein, 1990; Ferrari *et al.*, 1993), who show that methionine deficiency leads to neural tube defects in cultured rat embryos and that the teratogenicity of human sera in the whole embryo culture could be reversed by addition of methionine. Homocysteine-methionine metabolism has an unique function in metabolism of one-carbon compounds, namely by its metabolite SAM, which is an universal methyl group donor in transmethylation reactions. It has been suggested that decreased DNA methylation (Matsuda and Yasutomi, 1992; Li *et al.*, 1992) and reduced protein methylation (Coelho and Klein, 1990) are teratogenic mechanisms. The constant withdrawal of homocysteinyl moieties by incorporation of methionine into proteins and the use of SAM in polyamine synthesis dictate a need for constant replenishment of homocysteinyl moieties, preferably in the form of methionine. The embryo seems to economize in this respect by not expressing cystathionine-ß-synthase during this phase of gestation.

General Discussion

11

Summary and main conclusions

Homocysteine hypothesis

Folic acid-preventable NTDs are probably based on a genetic predisposition which is expressed as a folate related metabolic disorder. Due to the mild nature of this metabolic disorder it usually does not give rise to any clinical symptoms in the mother. However in combination with certain environmental factors it may affect the neurulation process of the embryo. Folic acid supplementation may overcome such a mild metabolic defect and in this way protect the embryo (Kirke *et al.*, 1993; Yates *et al.*, 1987; Scott *et al.*, 1994). Besides a low folate status in combination with a folate related metabolic defect several studies suggest that a low vitamin B12 status or a disordered transport or metabolism of vitamin B12 are associated with an increased risk for NTDs as well (Schorah *et al.*, 1980; Gardiki-Kouidou and Seller, 1988; Magnus *et al.*, 1991; Economides *et al.*, 1992; Kirke *et al.*, 1993; Mills *et al.*, 1995). It has therefore been suggested that a reduced substrate flux through methionine synthase is an important mediator in the aetiology of NTDs. A reduced substrate flux through methionine synthase will lead to an increase in homocysteine concentrations in blood and tissues, which are more pronounced after an oral methionine loading test. This has led to the hypothesis that these increased homocysteine levels, either maternal or in embryonic tissues, are responsible for the increased risk for a NTD-affected pregnancy. Investigation of the effects of increased homocysteine concentrations on the development of neurulating embryos was the main objective of this thesis. The culture of postimplantation rat embryos during the neur-

ulating period was the tool by which most of these investigations were performed.

In chapter 8 we investigated the embryotoxicity of homocysteine in gestational day 10 rat embryos in the postimplantation rat embryo culture. It was found that homocysteine was embryotoxic in a stereospecific way. L-homocysteine induced many malformations, however no NTDs. Even at the highest concentration tested (2.7 mM) D-homocysteine did not produce any embryotoxicity. The most frequent malformations that were caused by L-homocysteine were enlarged and transparent rhombencephalon, no or delayed fore limb bud formation, irregular and reduced number of somites and blisters - sometimes filled with blood - dorso-lateral of the place of fore limb bud formation. The lowest dose with an observable effect (reduced mitotic index of the neural epithelium and decreased mesenchymal cell density) was 0.9 mM and the lowest dose that induced morphological defects was 1.8 mM, which is 100-fold higher than the blood homocysteine levels observed in mild hyperhomocysteinemic women. In a subsequent study (chapter 9) in which gestational day 9.5 rat embryos were cultured in medium largely consisting of human serum and to which 1 or 2 mM L-homocysteine had been added, embryos developed normally, whereas embryos failed to close there neural tube when L-homocysteine was omitted. The high levels of homocysteine needed to induce embryotoxicity, the lack of induction of NTDs by L-homocysteine and even the prevention of NTDs by L-homocysteine in rat embryo culture make it highly unlikely that elevation of homocysteine concentrations either in the maternal serum or in the embryonic tissues is a causal factor in the aetiology of NTDs. Blood homocysteine levels elevated to a greater degree as compared to healthy controls after an oral methionine load should therefore only be interpreted as a biochemical risk indicator, showing homocysteine/methionine metabolism is not functioning optimally.

The mechanism of the embryotoxicity observed in cultured rat embryos after exposure to high levels of L-homocysteine was further investigated in chapter 9. Gestational day 11 rat embryos were exposed to L- and D-homocysteine. To be able to measure homocysteine-related metabolites in the embryonic tissues, older embryos were used in this experiment. It was found that exposure to 3 mM L-homocysteine increased embryonic SAH concentrations to such an extent that the SAM/SAH ratio was reduced to a level were inhibition of transmethylation reactions should be expected. We therefore proposed that this decrease of the SAM/SAH ratio and the subsequent inhibition of transmethylation reactions are intermediary in the mechanism of L-homocysteine embryotoxicity *in vitro*. This proposition is furthermore supported by the following observations: (i) D-homocysteine is much less embryotoxic (at the highest concentration tested (2.7 mM) no toxicity was observed), and (ii) the increase of embryonic SAH levels after D-homocysteine exposure is much smaller as compared to the increase observed after L-homocysteine exposure. These observations correlate with the enzyme

kinetic properties of SAH hydrolase - the enzyme converting homocysteine and adenosine to SAH, when homocysteine concentrations are high - *i.e.* the Km for D-homocysteine was 79 times as high as the Km for its enantiomere.

We also demonstrated homocysteine oxidizes to its disulfide homocystine - and probably also cysteine-homocysteine and protein-homocysteine mixed disulfides - when it is added to the embryo culture (chapter 8 and 9). Addition of transition metal ions enhances these oxidation reactions. The reduction of homocysteine embryotoxity by addition of transition metal ions and the absence of reduction of homocysteine embryotoxity when catalase is added make it unlikely homocysteine embryotoxicity is based on a H_2O_2 producing transition metal ion catalyzed oxidation of homocysteine in the medium as it was proposed for endothelial toxicity of homocysteine (Starkebaum and Harlan, 1986).

A study of the gestational development of homocysteine metabolizing enzymes in the rat (chapter 10) showed that cystathionine-ß-synthase activity first appears in the liver and therefore is not present in embryonic tissues before this organ is formed. Two enzymes of the methylation cycle that metabolize homocysteine (methionine synthase and SAH hydrolase) are present during the neurulation period indicating this cycle is active at this stage of development. Owing to the absence of cystathionine-ß-synthase activity in embryonic tissues during the neurulation period, the homocysteinyl moiety is conserved in the methylation cycle. The constant withdrawal of homocysteinyl moieties by incorporation of methionine into proteins and the use of SAM in polyamine synthesis dictates a need for continuous replenishment of homocysteinyl moieties, preferably in the form of methionine. The embryo seems to economize in this respect by not expressing cystathionine-ß-synthase during this stage of gestation. This feature of embryonic homocysteine metabolism constitutes another circumstantial evidence that slightly elevated homocysteine concentrations are more likely beneficial to embryonic development rather than the cause of deleterious effects.

Methionine hypothesis

Besides increased homocysteine levels due to decreased substrate flux through methionine synthase, decreased activity of methionine synthase will decrease the formation of methionine. In the experiment wherein homocysteine prevented neural tube defects in cultured rat embryos, methionine showed the same preventive effect at even lower concentrations (chapter 9). This suggested that the preventive effect of homocysteine might be mediated by providing the embryo with more methionine. The same study showed that folinic acid (5-formylTHF) could not attenuate the teratogenicity of the serum used. This suggests that prevention of NTDs in this model is much more linked with an adequate supply of methionine than with support of folate mediated purine and pyrimidine synthesis. Prevention of neural tube defects in cultured rat embryos

by methionine supplementation has been demonstrated before (Coelho *et al.*, 1989; Coelho and Klein, 1990; Flynn *et al.*, 1987). Also studies on antilaminin antibodies showed methionine to be a limiting nutrient for normal development of cultured rat embryos (Chambers *et al.*, 1995). Essien showed that methionine supplementation promoted the remediation of axial defects in *Axd* mutant mice (Essien, 1992; Essien and Wannberg, 1993a,b). Poor reproductive outcome in monkeys and humans could be improved by methionine supplementation (Klein *et al.*, 1993; Ferrari *et al.*, 1993). Although the latter studies are concerned with recurrent abortion and infertility, they still may be linked to NTDs since over 90% of NTD-affected embryos are lost by the end of the embryonic period and the estimated total prenatal mortality rate of NTD-affected embryos is 98.4% (Shiota, 1993).

Thus besides our own results presented in chapter 9, several other reports, especially those from Norman Klein and co-workers, provide evidence that methionine is the nutrient that is most limiting in the process of neural tube closure. The prevention of NTDs by folic acid supplementation may therefore be based on an increase of the methionine supply in embryonic tissues involved in neurulation. Folic acid supplementation will increase the availability of 5-methylTHF and in this way increase the substrate flux through methionine synthase. Consequently the supply of methionine will be increased. This process may be of importance both in the maternal and in the embryonic tissues. Increase of methionine formation in the maternal tissues will reduce the need of these tissues to depend on the nutritional supply of methionine and therefore less methionine will have to be withdrawn from the circulation. Increased blood methionine levels will lead to a more sufficient provision of this crucial nutrient to the embryo. Additionally increased 5-methylTHF serum concentrations will increase the ability of the embryonic tissues to remethylate homocysteine to methionine and therefore make them less dependent on the maternal methionine supply.

Finally there may be some special circumstances in the tissues involved in neurulation making them more prone to nutritional insufficiencies. These tissues divide rapidly and the nutritional demand will therefore be increased. Furthermore the blood supply, especially to the apical parts of the neural walls is limited. Nutrients might therefore easily become diluted while these tissues are increasing in biomass. Nutrient supply may therefore become critical in these tissues during neurulation and the one most limiting will be the one that first evokes a deleterious effect. When methionine is this most limiting nutrient, folic acid supplementation will prevent the developmental derailment by increasing both 5-methylTHF and methionine supply to the embryo.

Perspectives

On the premise that an insufficient methionine supply plays a pivotal role in the aetiology of folic acid-preventable NTDs, one may speculate on several aspects of the aetiology of NTDs and the prevention of a large proportion of them by peri-conceptional folic acid supplementation. One question is 'What are possible causes leading to an insufficient methionine supply to the embryonic tissues involved in neurulation and may therefore be causal factors in the aetiology of NTDs?'. Another question is 'Why is methionine so important for the neurulation process?'. Finally one could ask 'What are the implications for the prevention of NTDs?' These questions will be addressed in the following sections.

Environmental factors leading to an insufficient methionine supply

When the question is asked what factors may cause an insufficient methionine supply to the embryonic tissues, the concept of a multifactorial aetiology of NTDs makes its entry once again. Although in some cases just a single causative factor may lead to insufficient methionine levels, it is more likely that an ensemble of several factors will cause such an insufficiency. These factors may be of an environmental as well as of a genetic nature.

One set of environmental factors that may contribute to an insufficient supply of methionine are nutritional deficiencies. The most obvious one is a *low intake of methionine*. This may occur when the diet is low in protein or when the proteins consumed are low in methionine. Little attention has been given to such a condition in relation to the aetiology of NTDs thus far. In early observational studies it was noted that protein intake was higher in mothers from social classes I and II than in mothers of lower social classes (Smithells *et al.*, 1977). Diets low in protein were classified as 'inadequate' and such diets were associated with an increased risk for an NTD-affected pregnancy (Laurence *et al.*, 1980). Although low methionine intake may have contributed to the increased risk for a NTD-affected pregnancy, it will be difficult to isolate such a condition as a separate risk factor, since individuals that consume a poor diet that has a low protein content, have a lower intake of other dietary constituents, notably vitamins, as well (Smithells *et al.*, 1977).

Another nutritional factor that may contribute to an insufficient methionine supply is a *low intake of folates*. Since the methylation of homocysteine to methionine is dependent on a sufficient supply of 5-methylTHF, an inadequate nutritional supply of folates may hamper the salvation of the homocysteinyl moiety and therefore induce a methionine deficiency. Inadequate intake of folates has been given the most attention during recent decades and it is now a well established risk factor for NTDs (Kirke *et al.*, 1993).

The other vitamin on which methionine synthase is dependent, is vitamin B12. *Inadequate intake of vitamin B12* may therefore also contribute to an insufficient methionine supply to the embryonic tissues. A low vitamin B12 concentration in blood or amniotic fluid has been reported to be associated with NTD-pregnancies (Schorah *et al.*, 1980; Economides *et al.*, 1992) and was identified as an independent risk factor for NTDs (Kirke *et al.*, 1993). Recently Black *et al.* (1994) reported that the folate status of women in a rural area of Mexico was normal, but a low vitamin B12 status (<74 pmol/l) was found in 15% of pregnant women. Knowing that the prevalence of NTDs in Mexico is 1 in every 300 births (International Clearinghouse, 1991), these data may be a sign on the wall.

Another set of environmental factors that may lead to an insufficient supply of methionine, is exposure to xenobiotics that either may enhance the catabolism or clearance of methionine or of vitamins necessary for its formation, or block the formation of methionine directly or indirectly. One group of such xenobiotics are the *anticonvulsants*. Several of them, notably phenytoin and phenobarbital, reduce the levels of folate (e.g. Dastur and Dave, 1987; Dansky *et al.*, 1987; Hillesmaa *et al.*, 1983) and therefore may reduce the degree in which homocysteine is remethylated to methionine. Interesting in this respect is the report by Nosel and Klein (1992) in which they demonstrated valproate teratogenicity in rats could be reduced by maternal methionine supplementation. In an *in vitro* study Lucock *et al.* (1994) showed that carbamazepine reduced the rate of conversion of folic acid to 5-methylTHF by rat liver homogenate, which shows that this drug has the potential of reducing the availability 5-methylTHF.

Other drugs interfering with folate metabolism are folic acid antagonists (methotrexate, aminopterin, pyrimethamine, trimethoprim, sulphasalazine), As discussed in the introduction (chapter 7), due to the severity of the folate deficiency induced by folic acid antagonists, these drugs exert an abortifacient action when administered early in gestation rather than induce malformations. Cycloserine (used in tuberculosis treatment) and possibly oral contraceptives may have an anti-folate action as well (Lambie and Johnson, 1985).

Chronic ethanol consumption is another way by which the folate stores will get depleted. The mechanism by which ethanol exerts its anti-folate action is unclear. Several mechanisms have been proposed, including reduced intestinal absorption, diminished liver storage and increased faecal and urinary loss (Lambie and Johnson, 1985).

Another compound which would be expected to be very interesting with respect to the study of the necessity of methionine in the neurulation process is *nitrous oxide* (N_2O). N_2O inactivates methionine synthase by oxidation of the cobalamin moiety over a prolonged period (days rather than minutes) (Nunn and Chanarin, 1985). In the light of the methionine hypothesis inactivation of this enzyme might therefore be expected to induce NTDs. However teratogenicity

studies with this anaesthetic gas have been somewhat disappointing in this respect. In humans N_2O is strongly suspected of increasing the rate of spontaneous abortions among exposed operating room and dental suite personnel (Baden, 1985). In rodents it increases the rate of resorptions and induces both soft tissue and skeletal malformations. In postimplantation rat embryo culture N_2O induced growth retardation and malformations, most notably disturbances of the normal development of the left/right body axis (Fujinaga *et al.*, 1988; Baden and Fujinaga, 1991; Fujinaga, 1995). Thus there is a lack of a teratogenic specificity of N_2O with respect to NTDs which may be the result of the effects of N_2O on both methionine synthesis and folate metabolism. Due to the prolonged inactivation of methionine synthase N_2O may not only decrease methionine levels, but hamper folate metabolism as well for a period long enough and in a degree grave enough that it compromises thymidine and purine synthesis. In this respect it may be comparable to the action of classical antifolates like aminopterin and methotrexate, which are also known for their abortifacient action and teratogenic aspecificity. In a recent report Fujinaga and Baden (1994) argued N_2O teratogenicity was rather caused by a lack of methionine than by a decreased folate metabolism, since methionine could prevent N_2O induced teratogenicity and folinic acid could not. However their argument is not conclusive since the protective effect of methionine may at least partly be based on a regulatory mechanism. When methionine is administered, SAM levels will increase. High SAM levels will inhibit the activity of MTHFR. The lowering of 5-methylTHF levels, which would be the result of this feedback mechanism, would lead to a low flux through methionine synthase. Inactivation of methionine synthase by N_2O is believed only to occur when the enzyme is in a metabolically active state (Christensen *et al.*, 1994). Methionine may therefore have protected methionine synthase from N_2O induced oxidative damage instead of simply preventing a N_2O induced methionine deficiency.

Genetic factors leading to an insufficient methionine supply

Mild metabolic defects that do not cause any problems in the mother may cause an inadequate methionine supply to or in the embryo when several of them occur together or when they occur in combination with one or more of the above mentioned environmental factors. These metabolic defects may be divided into three groups: (i) Those affecting folate dependent enzymes, (ii) those that affect vitamin B_{12} metabolism, and (iii) those that affect methionine catabolism.

Yates *et al.* (1987) first proposed a disordered folate metabolism as a basis for genetic predisposition to neural tube defects. The hypothesis this could be due to a decreased activity of intestinal γ-carboxypeptidase - an enzyme that breaks down the polyglutamated folates to their absorbable monoglutamated forms -

was investigated by Bower *et al.* (1993). Their results did not support this hypothesis, but rather indicated that the distribution or metabolism of folic acid or its metabolites was impaired. Results of another study of limited scale (Lucock *et al.*, 1994) also suggested that some NTD-mothers had a biochemical lesion somewhere in the multi-enzyme pathway leading from folic acid to 5-methyl-THF. Very interesting in this respect are two independent observations. One is the finding that mild hyperhomocysteinemia occurs more frequently in NTD-mothers (25%) than in a control population (Steegers-Theunissen *et al.*, 1991; 1994). The other is the identification of a *thermolabile variant of MTHFR* which in its homozygous form leads to a reduced activity of this enzyme and causes mild hyperhomocysteinemia (Kang *et al.*, 1988). Subsequent studies at our university showed that in NTD-children and their mothers and fathers homozygosity for the thermolabile variant of MTHFR occurred in 14,16, and 10% of the cases, respectively (VanderPut *et al.*, 1995). Thus homozygosity for this mild enzymatic defect may be one of the forms of genetic predisposition for folic acid-preventable NTDs. Curiously it was found that in a control population the prevalence of homozygosity for thermolabile MTHFR was 5% (Kang *et al.*, 1991). This means that the gene frequency for this trait is very high in the general population. Such high gene frequencies usually occur only if there is some evolutionary advantage. Just like the high frequency for a genetic defect causing sickle cell anaemia in some African populations is explained by the increased resistance of affected individuals for malaria. It might be interesting to speculate on the rationale behind the high frequency of the thermolabile MTHFR trait. Reduced activity of MTHFR will result in a shift of the one-carbon flow from methionine synthesis to purine and thymidine synthesis. When our predecessors descended from the trees they changed their diet to staple foods containing less folic acid. In periods of food scarcity nutritional folate deficiency must therefore have occurred frequently. Severe folate deficiency will impair purine and thymidine synthesis and therefore decrease reproductive fitness of folate deficient individuals. Individuals inflicted with homozygosity for the thermolabile variant of MTHFR will however be protected from such an impairment to a certain degree, since their one-carbon flow is directed to purine and thymidine synthesis to a greater degree than in normal individuals. In other words their reproductive fitness will be greater under the conditions of a general folate deficiency. Surely such individuals would have an increased risk for an NTD-affected pregnancy, however this disadvantage might not counterbalance the advantage of a more efficient nucleotide synthesis. Thus increased purine and thymidine synthesis in individuals homozygous for thermolabile MTHFR may have resulted in a greater reproductive fitness in these individuals under conditions of general folate deficiency and this may have resulted in selection of the thermolabile MTHFR trait.

Another enzyme which is a likely candidate for a folate related mild enzymic defect leading an to insufficient methionine supply is *methionine synthase*.

Interesting results have been obtained by a group of Irish researchers (Kirke *et al*, 1993, Mills *et al*, 1995) They showed that in a group of NTD-mothers in which vitamin B12 plasma concentrations were in the lower range total homocysteine concentrations were higher than in healthy controls This effect was independent of the folate status of these women They suggested cases possessed an abnormal methionine synthase that has a reduced metabolic capacity when vitamin B12 levels are low, however in a range where the normal enzyme is fully functional and active This may be a plausible explanation for the results they obtained, however their results may be interpreted in other ways as well Firstly the defect may also be caused by a defect in vitamin B12 metabolism Mills *et al* (1995) showed there was no difference in methylmalonic acid values between controls and cases, indicating B12 metabolism is normal up to the level of the Co^{2+} form of cobalamin However, vitamin B12 still has to be processed a little further before it is a functional component of methionine synthase Thus defects in the cobalamin binding capacity of methionine synthase, the reduction of Co^{2+} to Co^{+}, or the methylation of the cobalamin moiety to its active methylcobalamin form are alternative explanations for the findings of Mills *et al* (1995) Several enzymes and cofactors are involved in these processes and the marked heterogeneity in the more severe forms of defective cobalamin metabolism at this level (cblG and cblE, Qureshi *et al*, 1994) indicates that the observations by Mills *et al* (1995) may also represent a heterogeneous population of mild enzymatic defects Secondly the results obtained by Mills *et al* (1995) and Kirke *et al* (1993) may just as well reflect the effects of mild enzymic defects in folate metabolism leading to reduced availability of 5-methylTHF This is because the crucial point seems to be the substrate flux through methionine synthase When 5-methylTHF availability is low the substrate flux through methionine synthase may still be sufficient when vitamin B12 levels are in the upper range and the amount of methionine synthase holo-enzyme therefore is large enough to compensate for the lower 5-methylTHF concentrations However when vitamin B12 levels are in the lower range the amount of methionine synthase holo-enzyme may be insufficient to compensate for the reduced 5-methylTHF availability Thirdly defects in the catabolic route of methionine may result in increased blood homocysteine and decreased blood methionine levels as well (see below)

A study by Schorah *et al* (1993) showed that incorporation of the methyl group of 5-methylTHF into DNA by trophoblasts derived from NTD-affected pregnancies was smaller than by trophoblasts derived from normal pregnancies They too suggested that the reduced activity of methionine synthase was the most likely explanation for this difference However they measured the incorporation of the carbon atom in thymidine For this the methyl group of 5-methylTHF first has to be oxidized to methylene This involves a multi-enzyme pathway of four enzymes consisting of both cytoplasmic and mitochondrial enzymes (Fig 7 4)

Thus far metabolic defects that lead to a reduced formation of methionine have been discussed. However an insufficient methionine supply may also be caused by increased catabolism of methionine. Methionine is catabolized through the transsulfuration pathway. The first three enzymes are enzymes of the methylation cycle. These are methionine adenosyltransferase (MAT), a specific methyltransferase used for catabolism of SAM: sarcosine synthase (or *N*-glycine methyltransferase, GNMT) and SAH hydrolase.

MAT is present in different isoforms. The predominant adult hepatic isoforms are MAT I and MAT III, which are probably coded by the same gene, but contain two and four α subunits, respectively (Kotb and Geller, 1993). MAT I has an intermediary Km for methionine (3-14 μM) and MAT III has a high Km for methionine (200 μM). The principal function of MAT III probably is catabolism of methionine when methionine levels are high. Reducing agents like dithiothreitol and glutathione (Pajares *et al.*, 1992a,b; Kotb and Geller, 1993) convert the MAT I isoform to the MAT III isoform. In extrahepatic tissues and in the fetal hepatic tissue the predominant form of MAT is MAT II. It consists of α-, β- and α-like (α') subunits. It has a low Km for methionine (6 μM). A less active MAT II-like isoform has also been demonstrated in human lymphocytes, which was designated as the λ-form. On mitogenic activation the λ-form is either converted to the more active MAT II isoform or is replaced by it. A number of studies indicate that the MAT II isozyme is the predominant or only active MAT in rapidly dividing tissues (Kotb and Geller, 1993). MAT III deficiency leads to hypermethioninemia. MAT II deficiency has not been described, probably because it is fatal. However changes in the regulation of the interconversions of different polymeric isoforms if existent may lead to pathological conditions (Kotb and Geller, 1993). Although possible changes in MAT activity in maternal and embryonic tissues are probably not related to a hyperhomocysteinemic condition, the relevance of such changes for the aetiology of NTDs may still exist, since SAM is the universal methyl group donor and the postulated pivotal role of methionine in neurulation may be through this metabolite.

When methionine levels are high the maternal liver will catabolize it with MAT III. The product of this reaction - SAM - is then further metabolized by GNMT. This enzyme is allosterically inhibited by 5-methylTHF. Hypothetically speaking a mutation leading to increased inherent activity of GNMT or decreased binding of 5-methylTHF to GNMT will lead to increased catabolism of SAM. Since SAM coordinates its own synthesis by its regulatory effects on cystathionine-β-synthase and MTHFR, increased remethylation and decreased transsulfuration of homocysteine will replenish the loss of SAM (Selhub and Miller, 1992). However when folate or vitamin B12 levels are low, the remethylating capacity of methionine synthase may be inferior to the metabolic demand. This would lead to mild hyperhomocysteinemia and hypomethioninemia. Therefore a derangement in GNMT activity might yet provide another alternative explanation to the observa-

tions made by Mills *et al.* (1995), who showed the blood homocysteine concentrations in NTD-mothers were elevated to a greater extent when their vitamin B12 levels were low, in comparison to the B12 and folate-matched controls. Given the fact that 5-methylTHF inhibits GNMT, aggravation of increased SAM catabolism under the condition of a folate deficiency may be anticipated. No reports are available on the activity of GNMT in NTD-mothers. The importance of this enzyme in the regulation of the steady state levels of SAM and the preservation of the homocysteinyl moiety in the methylation cycle make this enzyme another interesting candidate to look at when a folate-related genetic predisposition to NTDs is investigated.

Other factors affecting methionine supply to the embryo

Increased activity of GNMT may also be related to yet another subgroup of NTDs, that is the subgroup of diabetes-related NTDs. Diabetic mothers have a three times greater risk to have a NTD-affected child than healthy controls have (Campbell *et al.*, 1986). GNMT is under hormonal and nutritional control. Its activity will be increased after glucagon stimulation and under conditions of starvation. It is abundant in the periportal region of the liver and in the proximal convoluted tubules of the kidney, tissues both involved in gluconeogenesis (Yeo and Wagner, 1994). It may therefore be anticipated that in diabetic mothers an increased amount of methionine is metabolized to pyruvate through this pathway, which may lead to an insufficient methionine supply to the embryo.

Finally moderate auto-immune reactions of maternal antibodies to the embryonic yolk sac may affect the efficiency of the yolk sac to convey nutrients from the maternal circulation to the embryo. Carey and Klein (1989) have shown that antibodies to a basement membrane protein - laminin - were responsible for the embryotoxicity of sera from monkeys with a poor reproductive outcome. Immunization of monkeys with good reproductive histories with laminin resulted in embryotoxicity of their sera in rat whole embryo culture and failure of the monkeys to reproduce (Weeks *et al.*, 1989). Further analysis of the sera of immunized monkeys showed that the antibodies caused a reduction in the number of microvilli on the yolk sac cells and a reduction of cell size (Chambers *et al.*, 1995). Reduced nutrient uptake by the embryos was associated with increased binding of the antibodies to the yolk sac. Extensive binding of autoantibodies to the yolk sac and the subsequent reduction in the nutrient flow to the embryo will inevitably lead to embryonic loss. However moderate binding will only affect those nutrients which are most limiting. In such cases supplementation with these nutrients or their precursors may overcome such a mild nutrient block.

Methylation hypothesis

Concerning the mechanism by which methionine exerts its preventive effect with respect to NTDs, it has been suggested methionine might increase the activity of the methylation cycle, *i.e.* the constant interconversion of methionine and homocysteine with intermittent donation of a methyl group by SAM. Coelho and Klein (1990) showed that methionine deprived cultured rat embryos had reduced ratios of methylated amino acids to unmethylated amino acids in proteins of the neural tube. However, the ratio of monomethylarginine to arginine in proteins of the heart was lower in methionine supplemented embryos as compared to unsupplemented embryos. Matsuda and Yasutomi (1992) argued that 5-azacytidine induced NTDs in cultured rat embryos were caused by reduced DNA methylation. However, their conclusion was based on measurement of DNA methylation by immunohistochemical methods, which can only be a semi-quantative approach. Furthermore studies in our laboratory showed that high levels of homocysteine decreased the SAM to SAH ratio to such an extent that methyltransferase reactions must have been impaired. However no NTDs were observed (chapter 8 and 9).

These first approaches do not disqualify the hypothesis reduced methylation is involved in the aetiology of NTDs, but rather demonstrate that until now little effort has been made to investigate this hypothesis. Nevertheless other mechanisms by which methionine can prevent NTDs should be investigated as well. Besides acting as a methyl group donor through SAM, methionine is used in protein synthesis and SAM is a precursor in polyamine synthesis. Both reduced protein synthesis and perturbed polyamine synthesis could have a deleterious effect on embryonic development as well.

Implications for the primary prevention of neural tube defects

If a lack of methionine is the cause of failure of the process of neurulation, one might suggest the easiest way to prevent NTDs is supplementation with methionine. Although from the work discussed in this thesis it seems evident sufficient methionine is essential to the neurulation process, there are several reasons that argue against such a strategy. The most important reason is that from animal experiments it is clear that overconsumption of methionine is harmful to both the mother and the conceptus. Young rats achieved optimal growth when they were fed a diet containing 0.3% L-methionine. However diets containing 2% or more methionine resulted in a reduced growth rate compared to the 0.3% methionine supplemented rats (Benevenga and Harper, 1967; Cohen *et al.*, 1958). Pregnant rats fed a diet containing 1.6% L-methionine from gestational day 10 till gestational day 20 resulted in a threefold increase of resorptions and 4% of the fetuses had skeletal malformations (Kaemmerer and Zinat, 1975).

There is also a possibility that increasing the methionine content of the diet will result in an increased risk for premature vascular diseases, since higher levels of dietary methionine will increase plasma homocysteine concentrations as well (Guttormsen *et al.*, 1994). Although there is no proof that slightly elevated homocysteine concentrations are the cause for the vascular defects, at this time it is still generally believed that the increased homocysteine concentrations in mild hyperhomocysteinemic individuals are responsible for these defects (Green and Jacobsen, 1995).

Nevertheless it makes sense to ensure an adequate methionine intake by consuming a diet with sufficient methionine containing proteins. It has been estimated that the daily requirement for methyl groups in humans is 0.35 mmole/kg every 24 hours (Mudd et al., 1975; 1980) 0 3 mmole is derived from methionine, which is provided nutritionally or by remethylation of homocysteine. It was also estimated that the homocysteinyl moiety cycles approximately twice in the methylation cycle before it is transsulfurated to cystathionine, when methionine intake is normal. The daily requirement for methionine may therefore be estimated to be approximately 1.5 g/day for an adult. However a lower intake can be compensated by increased homocysteine remethylation and decreased transsulfuration under the condition folate metabolism is functioning normally. Supplementation with methionine bears the risk of overconsumption and should therefore only be considered under clinically controlled conditions.

Peri-conceptional supplementation with folic acid has been proven to be efficient in the prevention of NTDs and should therefore be part of a strategy for the primary prevention of NTDs.

A low vitamin B12 status has been shown to be an independent risk factor for NTDs (Kirke *et al.*, 1993). Subsequently Mills *et al.* (1995) showed that NTD-mothers who had a plasma B12 concentration in the lower range (<243 ng/l), had higher plasma homocysteine concentrations than controls that were matched for the B12 status, and this effect was found to be independent of the plasma folate concentration. Furthermore plasma and amniotic fluid vitamin B12 concentrations were lower in NTD-mothers as compared to controls (Economides *et al.*, 1992; Schorah *et al.*, 1980). These data suggest that an adequate B12 status may be as important as an adequate folate status for the prevention of NTDs. It makes therefore sense to ensure an adequate vitamin B12 status by vitamin B12 supplementation as well. Furthermore vitamin B12 supplementation may be especially relevant when folate supplementation is considered by the means of food fortification. With respect to the fortification of staple foods with folic acid in order to achieve an increased folic acid intake in the general population there has arisen considerable debate on the increased risk for the elderly to have neurologic disorders that are related to vitamin B12 deficiency (Wald and Bower, 1994) Vitamin B12 deficiency often presents first as pernicious anaemia. An increased intake of folic acid will prevent this pathologic

condition. However the neurologic disorders won't be prevented by increased folic acid intake. Thus there may be an increased risk that patients with an acquired myelopathy due to vitamin B12 deficiency first seek medical care when already irreversible neurologic damage has occurred. Adding extra vitamin B12 to the staple foods in conjunction with folic acid fortification will prevent such unwanted side effects and may in fact prevent acquired neurologic disorders and pernicious anaemia that are related to vitamin B12 deficiency.

Based on the present knowledge it can be concluded that the best strategy for the primary prevention of NTDs is consumption of a diet containing an adequate amount of methionine-rich proteins, supplemented with folic acid and vitamin B12.

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Dit proefschrift, getiteld **Embryotoxiciteitsstudies betreffende cyclofosfamide en homocysteine** bestaat uit twee delen. Deel I gaat over de verdere ontwikkeling van het ratte-embryo kweekmodel waarbij ten behoeve van de omzetting van lichaamsvreemde stoffen levercellen van volwassen ratten aan het embryo kweekmodel werden toegevoegd. In deel II wordt verslag gedaan van onderzoek naar de mogelijke rol van homocysteine in het ontstaan van neurale buisdefecten.

Deel I. Om de teratogeniteit - het vermogen om aangeboren afwijkingen te doen ontstaan - van stoffen te onderzoeken kunnen deze in het ratte-embryo kweekmodel getest worden. In dit model kunnen ratte-embryo's gedurende de periode van de orgaanaanleg buiten het moederdier in leven gehouden worden en gebruikt worden voor experimenten. Sommige stoffen zijn echter niet teratogeen van zichzelf, maar moeten eerst omgezet worden in een andere verbinding die wel teratogeen is. Een bekend voorbeeld van een stof die eerst omgezet moet worden, voordat deze teratogene effecten kan veroorzaken, is cyclofosfamide. Deze stof werd dan ook in onze experimenten gebruikt als een modelstof. De omzetting van lichaamsvreemde stoffen vindt hoofdzakelijk in de lever plaats. Het gekweekte ratte-embryo heeft echter nog geen lever ontwikkeld en de enzymen die nodig zijn voor de omzetting van de lichaamsvreemde stof zijn niet of slechts in zeer beperkte mate aanwezig in andere embryonale weefsels. Om toch de rol van dit soort omzettingen in het ratte-embryomodel te kunnen onderzoeken werd dit model gecombineerd met de kweek van levercellen afkomstig van volwassen ratten. Twee benaderingen werden hierbij onderzocht. In de eerste werden de levercellen rechtstreeks aan het serum waarin het ratte-embryo werd gekweekt, toegevoegd. In de tweede benadering werden de levercellen eerst gekweekt in een kunstmatig medium, waaraan de te onderzoeken stof werd toegevoegd. Vervolgens werden monsters van dit medium toegevoegd aan het serum waarin de embryo's werden gekweekt. Beide benaderingen bleken te voldoen, maar hebben ieder hun eigen voor- en nadelen, waardoor de precieze vraagstelling van een onderzoek zal bepalen welke van de twee benaderingen de meest geëigende is. Wanneer onze resultaten vergeleken werden met die van andere onderzoekers, bleek dat de gevoeligheid van ons model een stuk lager was dan die die zij rapporteerden. Nader onderzoek wees uit dat de combinatie van het gebruik van mannelijke levercellen - wij gebruikten levercellen van drachtige vrouwtjes - en een voorbehandeling van deze ratten met een PCB-mengsel - een methode om de omzetting van lichaamsvreemde stoffen te versterken - door de andere onderzoekers de oorzaak was van de gevonden discrepantie. Een opmerkelijk resultaat uit het vervolgonderzoek was dat de voorbehandeling van drachtige ratten met het PCB-mengsel nauwelijks een versterkend effect op de omzetting van cyclophosphamide had. Een goede verklaring hiervoor kon op grond van onze resultaten niet worden

gegeven. Onze onderzoekingen toonden in elk geval wel duidelijk aan dat wanneer levercellen van volwassen dieren met het ratte-embryomodel gecombineerd worden ten einde de omzetting van lichaamsvreemde stoffen in het onderzoek te betrekken, het van het allergrootste belang is wat de oorsprong van deze levercellen is. Dit geldt niet alleen voor het geslacht van de leverceldonoren, maar ook voor het soort voorbehandeling die de ratten hebben ondergaan.

Deel II. Spina bifida (open ruggetje) en anencephalie (kattekopje) zijn ernstige aangeboren afwijkingen welke veroorzaakt worden doordat vroeg in zwangerschap (4e post-conceptionele week) de neurale buis niet sluit. Zij worden dan ook neurale buisdefecten (NBD) genoemd. Het is gebleken dat ongeveer 70% van deze afwijkingen voorkomen kan worden door al voor de conceptie te starten met het innemen van extra foliumzuur, een vitamine uit de B-reeks. Het mechanisme van de beschermende werking van foliumzuur is echter onbekend. In deel II werd onderzoek gedaan naar dit mechanisme. De uitgangshypothese daarbij was de volgende. Wanneer er een tekort aan foliumzuur is, of wanneer er een stoornis is in de stofwisseling van dit vitamine dan zullen de homocysteineconcentraties in het lichaam stijgen. Homocysteine is een aminozuur in de stofwisseling van methionine. Constante omzetting van methionine in homocysteine en *vice versa* is van belang bij de overdracht van methylgroepen naar tal van belangrijke moleculen in het lichaam. Er werd verondersteld dat een verhoogde homocysteineconcentratie de sluiting van de neurale buis verstoort. Om deze hypothese te onderzoeken werden ratte-embryo's gedurende de fase van de sluiting van de neurale buis blootgesteld aan verhoogde homocysteineconcentraties. Het bleek dat sterk verhoogde homocysteineconcentraties weliswaar toxisch zijn voor het embryo, maar pas bij een concentratie die wel honderd maal hoger ligt dan die die bij vrouwen wordt gevonden die een kind met een NBD hebben gehad. Bovendien veroorzaakte de hoge homocysteineconcentratie geen NBD in het ratte-embryomodel. Het bleek zelfs dat wanneer ratte-embryo's werden blootgesteld aan hoge homocysteineconcentraties aan het begin van het proces van de sluiting van de neurale buis, dit het ontstaan van NBD's kon voorkomen, wanneer de embryo's in menselijk serum werden gekweekt. Methionine had hetzelfde preventieve effect bij een concentratie die een factor tien lager lag. Folinezuur (leucovorine, een derivaat van foliumzuur dat zonder voorafgaande reductie in de foliumzuurstofwisseling kan deelnemen) kon de NBD's echter niet voorkomen. Hieruit werd geconcludeerd dat het beschermende effect van homocysteine in het ratte-embryomodel en foliumzuur bij de preventie van NBD's verklaard moet worden doordat ze de beschikbaarheid van methionine vergroten. Een belangrijke consequentie van deze conclusie ten aanzien van de preventie van NBD's is, dat niet alleen een voldoende foliumzuurinname van belang is in het voorkómen van NBD's, maar dat ook een adequate vitamine B12 spiegel van groot belang is. Tevens dient het dieet

voldoende rijk te zijn aan eiwitten waarin het aminozuur methionine zit.

Curriculum vitae

De schrijver van dit proefschrift werd geboren op 4 september 1957 te Roermond. Na het behalen van het Atheneum B diploma in 1975 aan het Thomas a Kempis College te Arnhem volgde hij de Nieuwe Leraren Opleiding te Nijmegen. De tweedegraads bevoegdheid tot het lesgeven in de vakken biologie en aardrijkskunde verkreeg hij in 1982. Hij zette zijn studie voort aan de Katholieke Universiteit Nijmegen, waar hij in 1988 met genoegen voor het doctoraal examen biologie slaagde. Gedurende zijn doctoraal deed hij een hoofdvak dierfysiologie, waarbij hij onderzoek deed naar de regulatie van prolactinesynthese en -afgifte in de zoetwatertilapia Oreochromus mossambicus (Prof. Dr. S. E. Wendelaar Bonga). Gedurende een bijvak microbiologie deed hij onderzoek naar ferredoxine en hydrogenase in de methaanbacterie Methanosarcina barkeri (Prof. Dr. Ir. G. D. Vogels). Tijdens het bijvak toxicologie verrichte hij onderzoek naar de blootstelling van asfalteerders aan polycyclische aromatische koolwaterstoffen (Prof. Dr. P. H. Hendersson). Gedurende het doctoraal participeerde hij regelmatig in het biologieonderwijs als student-assistent. Vanaf maart 1988 is hij verbonden aan de Vakgroep Toxicologie aan de Katholieke Universiteit Nijmegen (Prof. Dr. J. Noordhoek) als docent en onderzoeker. Als docent is hij met name betrokken bij het onderwijs in de biochemische toxicologie en de reproduktietoxicologie. Het onderzoek dat werd verricht, is in dit proefschrift weergegeven. Hij is niet getrouwd en heeft geen kinderen.

Papers

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VanAerts LAGJM, Blom HJ, Copius Peereboom-Stegeman JHJ, Noordhoek J and Eskes TKAB Prevention of neural tube defects by L-homocysteine in cultured rat embryos Poster presented at *International conference on homocysteine metabolism, July 2-6, 1995, Dromoland Castle, Co Clare, Ireland* Published in *Irish Journal of Medical Science 1995,164(suppl 15) 22*

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increase of methionine supply? Some clues from postimplantation rat embryo culture experiments Oral presentation at *4th Symposium on vertebrate whole embryo culture, November 30 - December 2, 1994, Milan, Italy* Published in *Toxicology in vitro 1995,9 677-684*

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Nawoord

Een proefschrift komt tot stand door de samenwerking van velen. Ik wil dan ook allen die aan dit werk hebben bijgedragen van harte daarvoor bedanken. Een aantal van hen wil ik met name noemen.

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Stellingen behorende bij het proefschrift

Embryotoxicity studies on cyclophosphamide and homocysteine,

L.A.G.J.M. van Aerts, 1995

1. Embryotoxiciteit van L-homocysteine, zoals deze in vitro werd waargenomen, is van geen betekenis voor de marginaal verhoogde homocysteineconcentraties, zoals deze werden waargenomen bij een deel van de vrouwen die een kind met een neuraalbuisdefect hebben gehad.

   Dit proefschrift

2. Ten aanzien van neuraalbuisdefecten is milde hyperhomocysteinaemie niet een mediator, maar een risico-indicator.

   Dit proefschrift

3. Een homocysteinyldeficientie kan leiden tot open neuraalbuisdefecten.

   Dit proefschrift

4. Door geen homocysteine te transsulfureren, behoudt het neurulerende ratte-embryo alle homocysteinyleenheden voor de transmethyleringscyclus.

   Dit proefschrift

5. Het beschermende effect van foliumzuur ten aanzien van het voorkómen van neuraalbuisdefecten berust waarschijnlijk op een vergroting van de beschikbaarheid van methionine.

   Dit proefschrift

6. Wanneer het preventieve effect van foliumzuursuppletie berust op het opheffen van een beperkte transmethyleringscapaciteit, dan kan deze laatste bestreden worden met foliumzuursuppletie of met het realiseren van een dieet rijk aan methionine bevattend eiwit.

   Coelho and Klein, Teratology 1990;42:437-451

7. De wijde verspreidheid van het thermolabiele MTHFR gen bij mensen (25% is drager) duidt erop dat er een evolutionair voordeel mee behaald kon worden. Verschuiving van de one-carbon-flow van methioninesynthese naar nucleotidensynthese kan gedurende periodes van beperkte foliumzuurinname zo'n voordeel bieden.

   Kang et al., Am. J. Hum. Genet. 1991;48:536-545

8. Indien softwareproducenten voor de ontwikkeling van hun produkten minder zwaar zouden leunen op post-marketing surveillance, dan zouden er aanzienlijk minder 'buggy programs' op de markt verschijnen.

9. De tijdwinst die verkregen werd door de introductie van eenvoudige tekstverwerkers wordt volledig teniet gedaan door de tijd die er besteed wordt aan de uitgebreide mogelijkheden van hedendaagse tekstverwerkers.

10. Daar het stopsignaal bij spoorwegovergangen er in veel verdergaande mate toe leidt dat fietsers daadwerkelijk stoppen dan het geval is bij overige verkeerslichten, verdient het aanbeveling de overige verkeerslichten te laten knipperen en van bellen te voorzien.